Chronic activation of extracellular-signal-regulated protein kinases by phenylephrine is required to elicit a hypertrophic response in cardiac myocytes

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Extracellular-signal-regulated protein kinases (ERKs) are activated rapidly and transiently in response to phenylephrine (PE) and endothelin-1 (ET-1) in cardiac myocytes, but whether this is linked to the subsequent development of the hypertrophic phenotype remains equivocal. To investigate this, we examined the dependence of the hypertrophic response on the length of exposure to PE in neonatal myocyte cultures. In addition to the initial transient activation of ERKs (maximum at 5–10 min), PE (10 μM) induced a second, more prolonged peak of activity several hours later. The activity of a transfected atrial natriuretic factor–luciferase reporter gene was increased 10- to 24-fold by PE. This response was inhibited by the α_1-antagonist prazosin (100 nM) and by U0126 (10 μM) and PD184352 (1 μM), inhibitors of ERK activation, irrespective of whether these were added before or up to 24 h after the addition of PE. Prazosin had no effect on ET-1 (50 nM)-stimulated atrial natriuretic factor–luciferase activity. Protein synthesis was enhanced by $35 \pm 6\,\%$ by PE, and this was blocked by prazosin added 1 h after the addition of PE, but decreased only by half when added 8 h after PE. Similarly, PE (48 h) increased myocyte area by 49 % and this was prevented by prazosin added 1 h after PE, but decreased only by half when added at 24 h. These results demonstrate that prolonged exposure to PE is required to elicit alterations in gene expression, protein synthesis and cell size, characteristic of hypertrophied myocytes, and they confirm that the initial peak of ERK activity is insufficient to trigger hypertrophic responses.

Key words: extracellular signal-regulated kinase, gene expression, myocyte hypertrophy.

INTRODUCTION

Isolated cardiac myocyte preparations respond to a variety of hormones and growth factors by recapitulating many of the characteristics of hypertrophy *in vivo*, including increased myocyte size, enhanced RNA and protein synthesis and the re-expression of certain foetal genes such as atrial natriuretic factor (ANF) (reviewed in [1–3]). Hypertrophic agonists identified by these responses include G-protein-coupled receptor agonists [α_1-adrenergic agents, endothelin-1 (ET-1) and angiotensin II], growth factors (insulin-like growth factor 1, leukaemic inhibitory factor, fibroblast growth factors) and cytokines such as interleukin-1β. Recent efforts by numerous research laboratories have attempted to identify the intracellular signalling pathways through which the hypertrophic response is established. Given the plethora of agents that can induce a hypertrophic response in cardiac myocytes, it is not surprising that no signalling pathway has emerged as the key event and it is likely that different pathways assume primary importance under different conditions.

Of the intracellular signalling pathways proposed to be linked to the development of cardiac hypertrophy, much evidence supports a role for one or more of the mitogen-activated protein kinase (MAPK) cascades (reviewed in [4]). The MAPKs consist of three main subfamilies: the extracellular-signal-regulated protein kinases (ERKs), the c-Jun N-terminal kinases and p38MAPKs. Whereas the ERKs are primarily activated by G-protein-coupled receptor agonists and growth factors, maximum activation of the c-Jun N-terminal kinases and p38MAPKs is by cellular stresses such as ischaemia/reperfusion, osmotic shock and inhibitors of protein synthesis and, hence, these are collectively known as the stress-activated protein kinases. Although numerous studies have indicated an important role for the stress-activated protein kinases in the development of myocyte hypertrophy [5–11], the role of ERKs in the development of hypertrophy has been rather more equivocal. Several studies have demonstrated that constitutively overexpressed active components of the ERK cascade are capable of inducing morphological and transcriptional features of hypertrophy such as increased cell size, ANF expression and sarcomerogenesis *in vitro* [12–15]. It has also been shown that overexpression of the ERK upstream kinase, MAPK/ERK kinase (MEK) 1, induces a compensated hypertrophy in transgenic mice [15]. Furthermore, the hypertrophic response to phenylephrine (PE) can be prevented by the use of an ERK antisense [16] or by the inhibition of MEK with U0126 or Raf (an MEK kinase) with SB-386023 [17]. However, other studies have shown that the MEK inhibitor PD98059 does not prevent PE-induced hypertrophy [18] and that ATP and carbachol can induce ERK activation but not a hypertrophic response in cardiac myocytes [19]. Part of the controversy undoubtedly derives from the different models and techniques used and from the particular hypertrophic end point studied.

If ERK activation is an important part of the hypertrophic response, one aspect remains unclear. Previous studies have shown that after the addition of hypertrophic agonists such as PE and ET-1, ERK activation is rapid and transient, with a maximum activity after 5–10 min and a decrease in activity back to baseline by approx. 1 h [20,21]. For the protein kinase C-

Abbreviations used: ANF, atrial natriuretic factor; EGF, epidermal growth factor; ERK, extracellular-signal-regulated protein kinase; ET-1, endothelin-1; β-gal, β-galactosidase; LUX, luciferase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; PE, phenylephrine; PKB, protein kinase B; X-gal, 5-bromo-4-chloro-3-indoyl-β-galactopyranoside.

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activating phorbol ester, PMA, ERK activity remains longer but returns to basal levels within 3 h [21]. Thus the relationship between this rapid and transient increase in ERK activity and the various aspects of the hypertrophic response, which take many hours or days to develop, is uncertain. One possibility is that the rapid induction of ERK activity acts as a trigger to initiate the hypertrophic response and, once activated, the triggering response is no longer necessary. In the present study, we have examined this possibility by blocking the hypertrophic stimulus at different times after initiation and determining the effect on the subsequent hypertrophic response. Our studies indicate that the initial peak of ERK activity is insufficient to elicit a hypertrophic response but that a continuing ability to activate ERK is required for hypertrophy to develop. We also provide evidence for a second peak of ERK activity after the addition of PE which may more closely correlate with the hypertrophic phenotype.

EXPERIMENTAL

Materials

Sprague–Dawley rats were obtained from Harlan U.K. (Bicester, Oxon, U.K.). All laboratory chemicals were purchased from Sigma (Poole, Dorset, U.K.), VWR International (Lutterworth, Leics., U.K.), Calbiochem (Nottingham, U.K.) or Life Technologies (Paisley, Renfrewshire, Scotland, U.K.), unless otherwise stated. Collagenase was from Worthington (distributed by Lorne Laboratories, Reading, Berks., U.K.). PD184352 was obtained from Professor Philip Cohen (MRC Protein Phosphorylation Unit, University of Dundee, Dundee, U.K.).

Preparation and culture of neonatal rat ventricular myocytes

Myocytes were prepared from the ventricles of 1- to 3-day-old rats as described previously [12,22], using pre-plating to reduce contaminating non-myocytes. Cells were plated on to gelatin-coated 60 mm dishes at a concentration of 4×10^6 cells in 4 ml for Western blotting or 1×10^6 cells in 4 ml for transfections. Protein synthesis experiments were conducted in 35 mm dishes with 1.3×10^6 cells in 2 ml of medium. In one set of experiments, myocytes were purified by discontinuous Percoll density-gradient centrifugation, which produces cultures in which $> 95\%$ of the cells are myocytes [23]. Myocytes were incubated at 37 °C in a humidified atmosphere of 5 % CO_2.

Transfections

Myocytes were plated overnight and the medium changed to maintenance medium (Dulbecco's modified Eagle's medium/Medium 199 in a 4:1 ratio) with horse serum added to 4 %. After 3–4 h, myocytes were transfected with 5 μg of pANF638LΔ5′-luciferase (ANF-LUX) reporter plasmid [24] and 2 μg of pON249 [25], a β-galactosidase (β-gal) control plasmid, as described previously [10]. Plasmids were purified by poly(ethylene glycol) precipitation [26]. After overnight transfection, the cells were washed once with 10 % (v/v) horse serum in maintenance medium and then washed twice with maintenance medium before incubation in the presence or absence of agonists and antagonists at times and concentrations as indicated in the text and Figure legends. After a further 48 h, the cells were harvested and assayed for LUX and β-gal as described previously [12], except that LUX quantification was performed using a TD-20/20 Luminometer.

Western blotting

Myocytes (4×10^6 cells/60 mm dish) were incubated overnight in the maintenance medium and were then exposed to agonists and antagonists at concentrations and for times as indicated in the Figure legends before extraction. Myocytes were washed twice in PBS and then scraped into 150 μl of extraction buffer [12.5 mM Tris, 2.5 mM EGTA, 1 mM EDTA and 50 mM NaF (pH 7.6)], containing 5 mM dithiothreitol, 1 % Triton X-100 and the following protease inhibitors: 200 μM leupeptin, 10 μM *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E64), 120 μM pepstatin A and 300 μM PMSF. Samples were transferred to an Eppendorf tube, homogenized and the cell debris removed by centrifugation (10000 rev./min for 5 min at 4 °C). The supernatant was removed, 5 μl was used for Bradford protein assay [27] and the remainder was boiled with 0.33 vol. of sample buffer [10 % (w/v) SDS/13 % (v/v) glycerol/300 mM Tris (pH 6.8)/130 mM dithiothreitol/0.2 % Bromophenol Blue). Samples containing equal amounts of protein were separated by SDS/PAGE [6 % (w/v) acrylamide stacking gel and 10 % resolving gel] and electroblotted on to a PVDF membrane. Total ERK and phospho-ERK were detected in parallel blots using antibodies from Cell Signaling Technology (catalogue nos. 9101S and 9102) used at 1:1000 dilution. After incubation with a horseradish peroxidase-linked secondary antibody, bands were detected using a chemiluminescence method and exposure to Hyperfilm (Amersham International). The bands on the film were determined quantitatively by laser densitometry.

Protein synthesis measurements

Myocytes (1.3×10^6 cells/35 mm dish) were incubated in 2 ml of maintenance medium to which agonists/antagonists were added at times and concentrations as indicated in the Figure legends. Measurement of the rate of protein synthesis was then initiated by the addition of [^{3}H]phenylalanine to give a final specific radioactivity of 570–970 d.p.m./nmol of L-phenylalanine (variation between experiments). Myocytes were then incubated for a further 24 h before the dishes were rinsed three times with PBS and were then incubated on ice for 20 min with 1 ml of 10 % (w/v) trichloroacetic acid. The cells were then scraped from the dish and transferred with a further 1 ml trichloroacetic acid wash to a glass tube. As a carrier protein, 50 μl of 10 % (w/v) BSA was added. Precipitated protein was collected by centrifugation in a bench-top centrifuge (1000 rev./min for 5 min at 4 °C) and the pellet was washed four times with 5 % trichloroacetic acid. The final pellet was dissolved by the addition of 20 μl of 1 M NaOH and 1 ml of NCS tissue solubilizer (Amersham International). The mixture was then transferred to a scintillation vial with 8 ml of scintillant and counted.

Determination of cell size

Myocytes, plated at a density of 1×10^6 cells/dish, were transfected as described above and incubated with or without agonist for 48 h. The cells were washed twice with PBS and then fixed in 4 % (v/v) formaldehyde in PBS. The fixed cells were incubated with 5-bromo-4-chloro-3-indoyl-β-galactopyranoside (X-gal) solution [2.5 mM X-gal, 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$ and 2 mM $MgCl_2$ in PBS]. After incubation at 37 °C to allow the colour to develop, transfected (blue) cells were imaged using a video hardcopier attached to an inverted microscope. A graticule was used to record the image size. Pictures of 32 randomly selected cells were taken from each dish and the cell area was

measured using VIDS 3 software after tracing the cell outline into a computer.

Statistical analysis

Data are presented as means ± S.E.M. for the number of independent experiments shown in the Figure legends. Data were analysed by paired or unpaired Student's t test as appropriate, with $P < 0.05$ considered to be statistically significant.

RESULTS

Time course of ERK activation in cardiac myocytes

As has been documented previously (e.g. [19,20]), PE induced a rapid and transient activation of ERK1 and ERK2, with a peak of activity at 5–10 min and a return to basal levels within 1 h (Figure 1). Continuing the time course up to 24 h, we noted a second peak of ERK activity that appeared several hours after the first and was more prolonged. However, the time of onset and the intensity of this second peak were quite variable between myocyte preparations, as is reflected by the large error bars on the pooled data. Indeed, in some myocyte preparations, no discernible second peak was observed. The second peak of ERK activity was also peculiar to PE, since we have not observed such a peak in response to ET-1, which is equally as effective as PE in inducing acute ERK activation (results not shown). There was no significant change in total ERK protein throughout the 24 h time course.

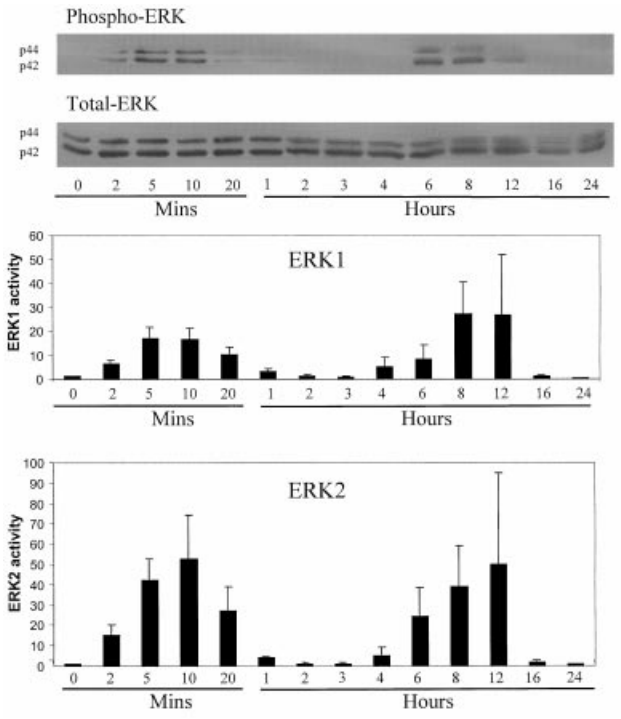

Figure 1 Extended time course of ERK activation after PE stimulation

After overnight incubation in the absence of serum and agonists, PE was added to a final concentration of 10 μM and cell extracts were prepared at the times indicated thereafter. Parallel Western blots were performed with equal amounts of total protein in each lane. ERK activity was determined by dividing the individual phospho-ERK bands by the equivalent total ERK band and expressing this relative to the control in the absence of PE. The data are means ± S.E.M. for three independent experiments. It should be noted that, because of the restrictions of size, samples 0–1 and 2–24 h were run on separate gels but were exposed on to the same photographic film. The image presented is a montage of the two, which explains the slight change in continuity between the 1 and 2 h samples. The control (0 h) sample was also run on the 2–24 h blot (results not shown) and used for its quantification.

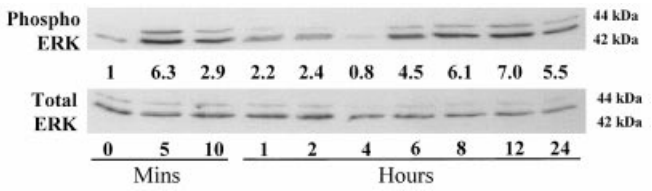

Figure 2 Time course of ERK activation in Percoll-purified myocytes

Myocytes were purified by Percoll gradient centrifugation as described in the Experimental section. After overnight incubation in the absence of serum and agonists, PE was added to a final concentration of 10 μM and cell extracts were prepared at the times indicated thereafter. Parallel Western blots were performed with equal amounts of total protein in each lane. The fold induction, shown below the phospho-ERK blot, was derived by dividing total (p42 and p44) phospho-ERK by total ERK and expressing this relative to the control in the absence of PE (time 0). The experiment was repeated and similar results were obtained.

The variability of the second peak of ERK activity in response to PE caused us to wonder whether it might be due to ERK activation in the population of non-myocyte cells that escape the pre-plating protocol, possibly associated with a particular phase of the cell cycle in these dividing cells. To test this possibility, myocytes were purified by Percoll density-gradient centrifugation, a procedure that results in highly purified myocytes [23]. Treatment of these Percoll-purified myocytes with PE initiated a biphasic response (Figure 2) similar to that in the less-homogeneous myocyte preparation (Figure 1). Thus the second peak of ERK activity is myocytic in origin.

Previously, it has been shown that treatment of cardiac myocytes with 5 μM ATP induces ERK activation but does not induce a hypertrophic response in these cells [19]. In the light of our observations mentioned above, we considered whether this might reflect a difference in the ERK activation profile compared with PE and, therefore, we compared the time courses of ERK activation in response to 10 μM PE and 5 μM ATP (Figure 3). Whereas ATP and PE induced similar early peaks of ERK activity, only PE induced a significant second peak of ERK activity. Thus this second peak of ERK activity may be more crucial to the hypertrophic response than the first peak.

Inhibition of PE-induced ANF-LUX expression by prazosin, U0126 and PD184352

Treatment of cardiac myocytes with the α_1-adrenergic antagonist, prazosin, induced a dose-dependent decrease in ANF-LUX expression in response to 10 μM PE (Figure 4A). Activation of ANF-LUX was decreased from 24.8-fold in the absence of prazosin to 3.3-fold in the presence of 100 nM prazosin. Thus inhibition was approx. 90 % complete at 100 nM prazosin and additional increases in concentration did not reduce PE-induced ANF-LUX expression further (3.4-fold at 1 μM prazosin). As a control for the specificity of prazosin, we examined its effects on ET-1-induced ANF-LUX expression. ET-1 induced a 14.3-fold increase in ANF-LUX expression and this was completely unaffected by prazosin at concentrations from 1 to 300 nM (Figure 4A). However, 1 μM prazosin decreased ET-1-stimulated ANF-LUX expression by about one-third. Hence, a concentration of 100 nM prazosin was chosen to provide selective and maximal inhibition of PE-induced ANF-LUX expression.

To establish whether 100 nM prazosin was effective in suppressing PE-induced activation of ERKs, myocytes were incubated

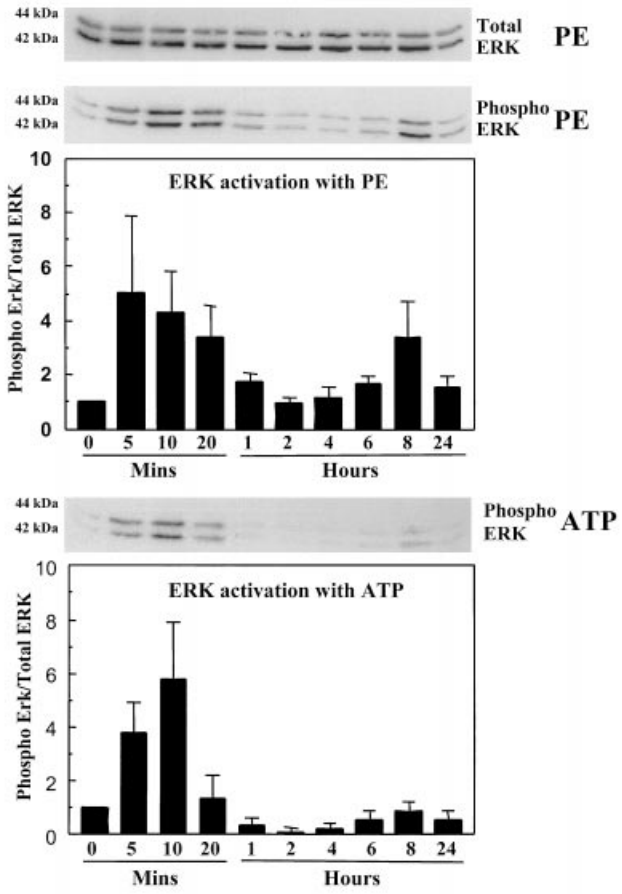

Figure 3 Comparison of the effects of PE and ATP on ERK activation

After overnight incubation in the absence of serum and agonists, myocytes were treated with 10 μM PE or 50 μM ATP for the indicated time period before extraction. Western blotting was performed as described in the Experimental section. Total phospho-ERK was divided by total ERK and expressed relative to the control in the absence of agonist. The data are means $\pm$ S.E.M. for three independent experiments.

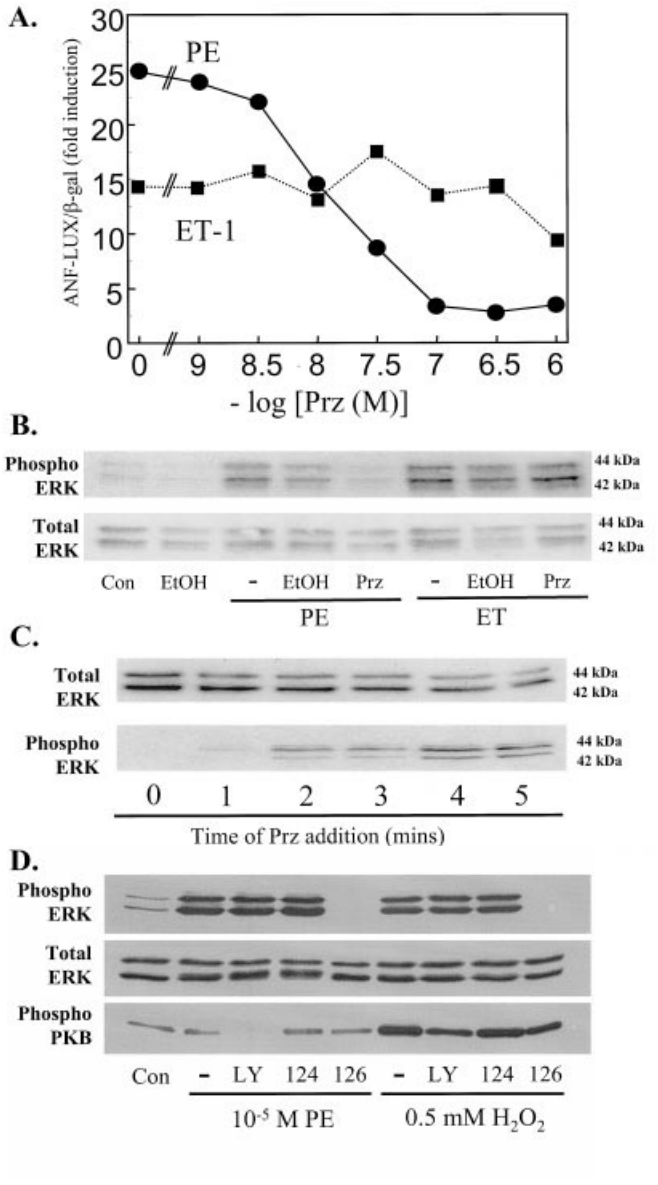

Figure 4 Effects of prazosin on PE- and ET-1-induced ANF-LUX expression and ERK activation in cardiac myocytes

(**A**) Dose–response curve for the effect of prazosin (Prz) on ANF-LUX expression. Myocytes were transfected with 5 μg of ANF-LUX and 2 μg of β-gal control plasmid as described in the Experimental section. Individual dishes were then treated with prazosin at the concentrations shown for 30 min before further incubation for 48 h in the presence or absence of 10 μM PE or 50 nM ET-1. Results are from a single experiment performed in duplicate and are expressed as ANF-LUX/β-gal ratios relative to controls performed in the absence of agonist or antagonist. (**B**) Effect of prazosin on ERK activation. Myocytes were pretreated for 10 min with prazosin (100 nM), ethanol (EtOH) or were left untreated before the addition of PE (10 μM) or ET-1 (50 nM), as indicated. ERK activity was assessed after a further 10 min. Results are from a single experiment which was repeated and similar results were obtained. (**C**) Time dependence of prazosin addition. Myocytes were incubated with PE (10 μM) for the indicated time period before the addition of prazosin (to 100 nM). ERK activation was assessed at 10 min after the initial PE addition. (**D**) Effect of U0126 on ERK activation. Myocytes were pretreated for 15 min with 10 μM LY294002 (LY), 10 μM U0124 (124) or 10 μM U0126 (126) or were left untreated (–) before the addition of PE or H_2O_2, as indicated. After a further 5 min, myocytes were extracted and assayed for phospho-ERK, total ERK and phospho-PKB.

with 10 μM PE for 10 min after 10 min preincubation in the presence or absence of 100 nM prazosin (Figure 4B). As expected, prazosin inhibited ERK activation by PE but did not affect ERK activation in response to ET-1. Ethanol, the vehicle for prazosin, had no effect on ERK activation. To determine how rapidly 100 nM prazosin was capable of suppressing PE-induced activation of ERKs, myocytes were stimulated with 10 μM PE for 10 min and prazosin was added at different times throughout this period. When prazosin was added co-incidentally with PE, ERK activation was completely suppressed (Figure 4C). Even when added 1 min after PE, prazosin was capable of almost completely suppressing PE-induced activation of ERKs. However, when cells were exposed to PE for 2 min or more before prazosin addition, little effect of prazosin was observed. These results demonstrate that 100 nM prazosin is capable of suppressing rapidly and completely the ERK activation by 10 μM PE. Furthermore, 100 nM prazosin is specific since it does not inhibit ERK activation or ANF-LUX expression in response to ET-1. The efficacy of the MEK inhibitor U0126 in inhibiting ERK activation was also established (Figure 4D). ERK activity in the presence of PE or H_2O_2 was abolished by U0126 but was unaffected by the inactive analogue U0124 or by the phosphoinositide 3-kinase inhibitor LY294002. Selectivity of U0126 was determined by examining protein kinase B (PKB) phosphorylation. Neither U0126 nor U0124 altered PKB phosphorylation,

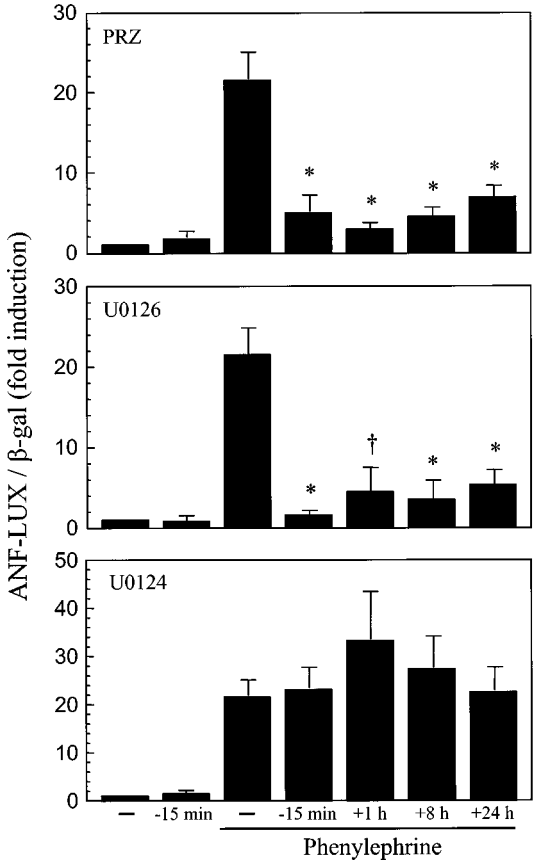

Figure 5 Time-dependent effects of prazosin and U0126 on PE-induced ANF-LUX expression in cardiac myocytes

Myocytes were transfected with 5 μg of ANF-LUX and 2 μg of β-gal control plasmid as described in the Experimental section. Individual dishes were then treated with 10 μM PE (zero time) or left untreated, as indicated. Prazosin (100 nM, top panel), U0126 (10 μM, middle panel) or U0124 (10 μM, bottom panel) were added 15 min before (—15 min) or 1, 8 or 24 h after treatment with PE. Cells incubated in the absence of inhibitor are indicated (–). Myocytes were incubated for a period of 48 h after the addition of PE, and results are expressed as ANF-LUX/β-gal ratios relative to controls performed in the absence of agonist or antagonist from five separate experiments. *$P < 0.005$, †$P < 0.01$ versus PE in the absence of inhibitor.

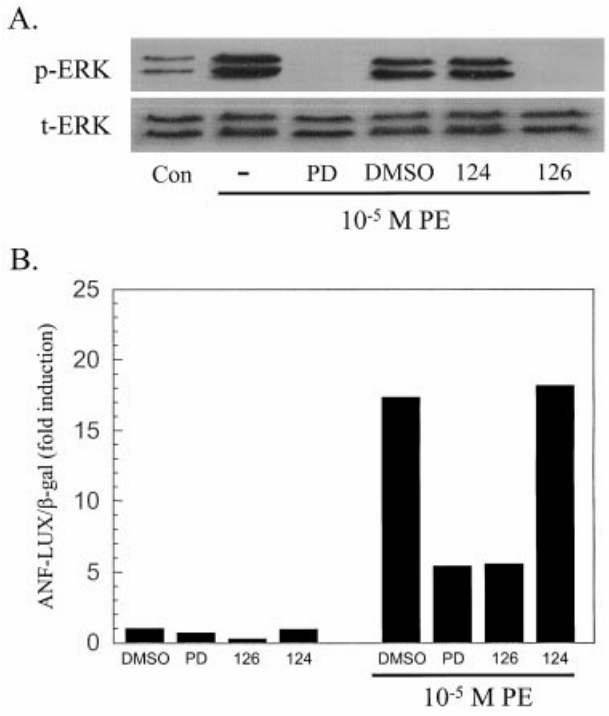

Figure 6 Inhibition of PE-induced ERK activation and ANF-LUX expression in cardiac myocytes by PD184352

(**A**) Effect of PD184352 on ERK activation. Myocytes were pretreated for 10 min with PD184352 (1 μM), U0124 (10 μM), U0126 (10 μM) or DMSO or were left untreated (–) before the addition of PE (10 μM), as indicated. ERK activity was assessed after a further 5 min by Western blotting. Results are from a single experiment. p-, phospho-; t-, total-. (**B**) Effect of PD184352 on ANF-LUX expression. Myocytes were transfected with 5 μg of ANF-LUX and 2 μg of β-gal control plasmid as described in the Experimental section. Individual dishes were then treated with DMSO (0.1%), PD184352 (1 μM), U0126 (10 μM) or U0124 (10 μM) for 15 min before the addition of PE as indicated. Myocytes were incubated for a further 48 h before assay. Results are expressed as ANF-LUX/β-gal ratios relative to DMSO controls and are the means for two separate experiments.

whereas LY294002 blocked PKB phosphorylation in the presence of PE and decreased it in the presence of H_2O_2.

To establish whether a short-term exposure to PE (which is capable of fully activating ERKs) is sufficient to trigger a hypertrophic response, myocytes were incubated for 48 h with PE, with addition of prazosin at different times (Figure 5, upper panel). In this series of experiments, PE induced a 20.8 ± 3.3-fold activation of ANF-LUX expression ($P < 0.005$) and, as before, prior treatment with prazosin almost completely prevented the response to PE. Interestingly, a similar degree of inhibition of ANF-LUX expression was observed when prazosin was introduced at 1, 8 or 24 h after PE had been added. Thus the stimulation of ANF-LUX expression by PE requires a constant input from the α_1-adrenergic receptor and there appears to be no trigger response or accumulated signalling memory.

Activation of the ERK pathway is not the only intracellular route through which PE may induce its hypertrophic effects. To establish more specifically whether constant signalling through the ERK pathway is necessary to mediate the hypertrophic response to PE, the MEK inhibitor U0126 was used (Figure 5, middle panel). Like prazosin, treatment of cells with U0126 inhibited the stimulation of ANF-LUX expression by PE, and the extent of inhibition was similar whether U0126 was added before or up to 24 h after the addition of PE. As a control for the effects of U0126, we examined the effects of U0124, an analogue of U0126, which does not interfere with MEK activity (Figure 5, bottom panel). U0124 had no effect on the ability of PE to stimulate ANF-LUX expression, irrespective of the time of its addition. Thus these experiments clearly demonstrate the need for continual ERK activity for the stimulation of ANF-LUX expression by PE.

In addition to specific effects on the activation of genes such as ANF that are indicative of a hypertrophic response, PE elicits a much lesser, but general, up-regulation of transcription and translation [28]. Notably, although the effects of prazosin and U0126 on ANF-LUX expression were similar, U0126 had a lesser effect when compared with prazosin on the expression of the co-transfected β-gal control plasmid. Thus, in this series of experiments, PE stimulated β-gal expression to $205 \pm 13\%$ of control ($P < 0.005$), and this was decreased to $116 \pm 20\%$ ($P < 0.01$) by prazosin but remained at $172 \pm 12\%$ ($P < 0.01$ versus prazosin) in the presence of U0126. The control compound U0124 did not affect PE-stimulated β-gal expression ($216 \pm 30\%$).

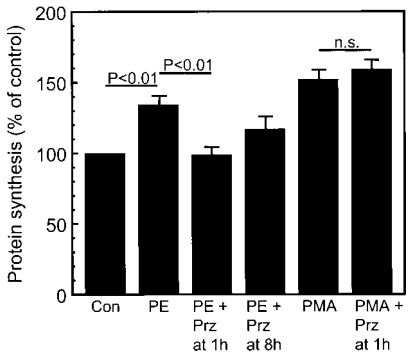

Figure 7 Prazosin inhibits myocyte protein synthesis in response to PE but not PMA

Myocytes were incubated in the presence or absence of 10 μM PE or 1 μM PMA before the addition of prazosin to a final concentration of 100 nM at the indicated times. After the addition of prazosin at 8 h, [^{3}H]phenylalanine was added to each dish, which was then incubated for a further 24 h before harvesting and assay for protein-bound [^{3}H], as described in the Experimental section. Results are expressed relative to the controls and are the means $\pm$ S.E.M. for four myocyte preparations. n.s., not significant.

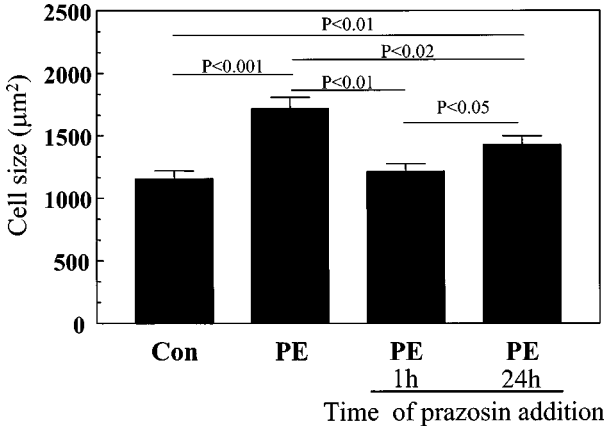

Figure 8 Initial ERK activity is insufficient to stimulate an increase in myocyte size

Myocytes were transfected with 5 μg of ANF-LUX and 2 μg of β-gal control plasmid as described in the Experimental section. After overnight incubation without serum, myocytes were incubated in the presence or absence of 10 μM PE for a further 48 h with addition of prazosin to 100 nM at the indicated times. Cells were fixed and stained with X-gal and the areas of 32 randomly selected transfected cells were quantified as described in the Experimental section.

This result suggests that the general effects of PE on transcription/translation are less dependent on ERK activity when compared with the specific effects such as induction of ANF expression.

Although U0126 has traditionally been used as an inhibitor of the ERK1/2 kinase MEK-1, activation of ERK5 by epidermal growth factor (EGF) in PC12 and HeLa cells is also inhibited by U0126 with similar potency [29,30]. Since the MEK-5–ERK5 pathway can induce a hypertrophic response in cardiac myocytes, including the induction of ANF expression [31], it was possible that our effects of U0126 were mediated through ERK5 inhibition rather than, or in addition to, inhibition of ERK1. To investigate this possibility, we used another compound, PD184352, at a concentration (1 μM) that inhibits ERK1/2 activation but does not affect ERK5 activation [30]. At this concentration, PD184352 was as effective as 10 μM U0126 in inhibiting PE-induced ERK1/2 activation (Figure 6A) and ANF-LUX expression (Figure 6B), indicating that the effects we have observed most likely involve MEK-1–ERK1/2 rather than MEK-5–ERK5.

Effect of timed prazosin addition on PE-induced protein synthesis

To determine whether short-term stimulation of myocytes with PE is sufficient to elicit an increase in the rate of protein synthesis, myocytes were treated with PE and prazosin, added 1 h or 8 h later. Protein synthesis was measured by determining [^{3}H]phenylalanine incorporation over the 24 h period following 8 h of PE stimulation (i.e. immediately after the later addition of prazosin) (Figure 7). Addition of 10 μM PE alone increased protein synthesis by $35 \pm 6\%$. This response was completely blocked by the addition of 100 nM prazosin, added 1 h after PE. If, however, prazosin was added after 8 h, PE-stimulated protein synthesis was inhibited by approx. 50% ($17 \pm 8\%$ increase). In contrast, stimulation of protein synthesis by the phorbol ester PMA was unaffected by the addition of prazosin at 1 h, demonstrating that there were no non-specific effects of prazosin on protein synthesis. These results show that continual exposure to PE is required to elicit its maximal effects on protein synthesis, but that there are important events that occur during the first few hours of PE stimulation that give rise to long-term effects on protein synthesis.

Effect of timed prazosin addition on PE-induced increases in cell size

Another aspect of the hypertrophic response to PE in cardiac myocytes is increased cell size. Myocytes exposed to 10 μM PE for 48 h exhibited a 49% increase in cell area (Figure 8). Treatment with 100 nM prazosin at 1 h completely prevented the response to PE, whereas treatment with prazosin after 24 h reduced the increase in myocyte area to 24%. This was still a significant increase, but also a significant decrease from PE alone. These results show that brief exposure to PE is insufficient to increase cell area, in accord with the observation that protein synthesis is not stimulated under these conditions.

DISCUSSION

The objective of the studies reported here was to determine whether a brief exposure to PE, sufficient to activate the ERK cascade fully, was capable of inducing a hypertrophic response in cardiac myocytes. Previous studies have reported that hypertrophic agonists induce a rapid, but transient, activation of ERKs, with a maximum response at 5–10 min [19–21,32]. Given that not all agonists that activate ERKs induce a hypertrophic response [19] and that hypertrophic responses occur over a more prolonged time course, the relationship of this early peak of ERK activity to hypertrophy is not easily established. In most of the studies, we chose to use prazosin to inhibit the response to PE for two main reasons. First, PE activates multiple intracellular signalling pathways downstream from the α_1-adrenergic receptor and not just the ERK pathway. Secondly, the lack of non-specific or toxic effects could be checked by retention of the response to other hypertrophic agonists.

In conducting a prolonged time course, we observed a second and much more delayed peak of ERK activity in response to PE (Figures 1–3). The time of onset, intensity and duration of this second peak varied much more when compared with the first peak and did not always coincide with the time points chosen in particular experiments, making it difficult to study. This may be one explanation for the failure to observe a second peak of ERK activity in response to PE in the study by Post et al. [19], who examined no time point between 6 and 18 h and conducted the time course study only twice. The extent of activation of ERKs in the acute phase after PE addition was also modest in their experiments (approx. 2.5-fold), rendering the observation of a second, more diffuse peak difficult. Other possible discrepancies between their study and ours include the use of a 10-fold higher PE concentration in their experiments, which might induce a more profound receptor down-regulation, and different assays for ERK activation. In the present study, we used Western blotting with antibodies specific to the dually phosphorylated forms of ERK to assess its activity, whereas in the study by Post et al. [19] ERK activity was measured directly using an in-gel assay with myelin basic protein as substrate. Thus it is possible, at least formally, although we consider it very unlikely, that the second peak we observed represents ERK that is dually phosphorylated but inactive.

A further disparity between the present study and that of Post et al. [19] is that we observed attenuation of PE-induced ANF-LUX expression after MEK-1 inhibition, whereas Post et al. did not. This is probably due to the efficacy and selectivity of the inhibitors used, since we have also failed to observe any inhibitory effect of PD98059, the inhibitor used in the study by Post et al. [19], on ANF-LUX induction (results not shown). However, interpretation of the effects of PD98059 is complicated by the observation that it has profound effects on basal ANF-LUX expression. In our experiments, basal ANF-LUX expression could be induced up to 7-fold by PD98059 in some instances, and there is evidence of a 2-fold activation of basal activity in the study by Post et al. [19]. The two inhibitors used in the present study, U0126 and PD184352, do not activate basal ANF-LUX expression (Figure 6), and, since they produce similar degrees of inhibition of PE-induced ANF-LUX expression, we are confident that ERK1/2 activation is an important component of this response. However, we imply no criticism of the Post et al. study, since these inhibitors were unavailable at the time of their study and their conclusions were also supported by the use of dominant-negative ERK1/2 constructs.

The use of inhibitors (U0126, SB-386023) has implicated the ERK cascade in PE- and ET-1-induced increases in cell size, sarcomeric organization and ANF expression [17]. However, in those studies the acute and chronic effects of ERK activation were not investigated because the inhibitors were added before agonist challenge. In the present study, we added U0126 and prazosin at various times after the addition of PE to discriminate between the importance of short- and long-term ERK activation. Our results, together with those of Post et al. [19], clearly demonstrate that transient ERK activation alone is insufficient to elicit a hypertrophic response. However, given that U0126 blocks ANF expression even when added long after the initial peak of ERK activity has subsided, the implication is that there is an ongoing requirement for ERK activation, at least in this aspect of the hypertrophic response. From our observation that PE induces a second, albeit variable, peak of ERK activity, we speculate that it is this activity, perhaps in addition to the initial peak, that is required for the hypertrophic response. Although this agrees with the observation that ATP does not produce this second peak (Figure 3) and is not a hypertrophic agonist [19], we failed to detect such a second peak of ERK activity with ET-1 both in the present study and in previous work [32]. However, it would be difficult to detect a modest increase in ERK activity that might manifest itself over a prolonged time.

The conclusion to be drawn from our results and those of others is that ERK activation plays an important role in the establishment of the hypertrophic response. The initial peak of ERK activity is not sufficient to initiate a hypertrophic response but may, nevertheless, be necessary. Moreover, it is probable that different aspects of the hypertrophic response may be more or less dependent on acute or chronic ERK activation and this may also vary with particular agonists. For example, PE activates ANF expression to a greater extent than does ET-1 (see e.g. Figure 4A) and this correlates with the observation that PE, but not ET-1, induces chronic ERK activation. On the other hand, ET-1 is more potent than PE in inducing c-*jun* transcription, which is also ERK-dependent [33]. Since c-*jun* is an immediate-early gene, its regulation is likely to be dependent on acute ERK activation, for which ET-1 is at least as effective as PE. Another example is PMA, which activates protein synthesis at least as well as PE (Figure 6) but is less effective in inducing ANF expression. PMA induces a more sustained initial peak of ERK activity when compared with either PE or ET-1 [34].

The biphasic nature of ERK activation has been observed in other cell types (see e.g. [35]) and the timing and intensity of ERK activation appears to be a critical determinant of cell fate [36,37]. Thus stimulation of endogenous EGF receptors induces transient ERK activation and proliferation in PC12 cells, whereas overexpression of the EGF receptor and subsequent agonist challenge leads to sustained ERK activation and differentiation [38]. In contrast, stimulation of PC12 cells with nerve growth factor leads to sustained ERK activation and differentiation. However, PC12 cell lines, which have decreased receptor number and which do not differentiate in response to nerve growth factor can be selected and these are found to exhibit only transient ERK activation [39]. It is also clear that the relationship between ERK activation and growth response is dependent on tissue type, an additional complexity, because in fibroblasts sustained ERK activation is associated with proliferation and not differentiation [40–42]. The differential response to transient versus sustained ERK activation may reflect compartmentalization of the signal, since evidence from PC12 cells suggests that sustained activation of ERKs is required for their translocation to the nucleus [36,38,43]. However, it is clear from other studies [44] that transient ERK activation can induce very rapid nuclear responses, such as the phosphorylation of ternary complex factor and subsequent c-*fos* transcription. Moreover, transient ERK activation has been shown to be necessary and sufficient for c-Fos protein expression in Rat-1 fibroblasts, whereas sustained ERK activation is required for the induction of some other activator protein 1 family members [45]. In terms of the hypertrophic response, the exact role of ERK is still not clear, but one possible model is that both immediate-early genes that are dependent on transient ERK activation and delayed gene expression dependent on sustained ERK activation are required.

In addition to characteristic changes in gene expression (such as ANF and c-*jun*), the hypertrophic response is accompanied by a general up-regulation of transcription/translation, resulting in increased ribosome number, enhanced protein synthesis and greater cell size. Results of our experiments during the present study indicate that these more global aspects of the hypertrophic response are less dependent on ongoing ERK activation than is ANF expression. Thus addition of prazosin after 8 or 24 h exposure to PE only inhibited subsequent increases in protein synthesis and cell size respectively by about half, compared with

a very much greater inhibition of ANF expression. Also, in transfection experiments, the co-transfected constitutively active β-gal reporter gene, whose expression is a crude measure of general transcription/translation, was much less affected by U0126 than by prazosin, implying less dependence on ERK activation. This conclusion is in agreement with a previous study [28] using effector mutants of V12Hras, which suggested that global effects on transcription may be mediated through non-ERK-dependent pathways.

The exact significance of the second peak of ERK activity in cardiac myocytes is difficult to ascertain, because it is not possible to eliminate the first peak but retain the second peak. Also, it seems clear that this second peak of ERK activity is not a uniform response to stimulation by hypertrophic agonists, since we have not observed such a response to ET-1. However, it is difficult to reconcile the ERK dependence of ET-1-stimulated hypertrophy [17] with the lack of a prolonged effect of ET-1 on ERK activity, since transient ERK activation is clearly insufficient to initiate a hypertrophic response. Probably, at least the observed basal levels of ERK activity are required for hypertrophic responses to be elicited. It is noteworthy, in passing, that biphasic ERK activation in cardiac myocytes with a less intense, but more prolonged, second peak of ERK activity has been reported recently in response to oxidative stress [46]. Thus it is possible that the extent to which hypertrophic agonists promote chronic ERK activation in cardiac myocytes may be related to the degree of oxidative stress that they induce.

In summary, we have demonstrated that exposure of neonatal rat myocytes to PE leads to a biphasic activation of ERKs with a rapid and short-lived acute response and a more protracted but delayed secondary response. The initial peak of ERK activity is insufficient on its own to elicit a hypertrophic phenotype but may contribute to it by priming the system. The second peak of ERK activity in response to PE may contribute to the hypertrophic response, and probably has a particular role in the stimulation of gene expression. The extent to which this second peak of ERK activity is observed in response to different agonists may contribute to the variations in activation of the different aspects of the hypertrophic response.

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