Ca^{2+}-independent protein kinase C activity is required for α_1-adrenergic-receptor-mediated regulation of ribosomal protein S6 kinases in adult cardiomyocytes

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The α_1-adrenergic agonist, phenylephrine (PE), exerts hypertrophic effects in the myocardium and activates protein synthesis. Both Ca^{2+}-dependent protein kinase C (PKC, PKCα) and Ca^{2+}-independent PKC isoforms (PKCδ and ε) are detectably expressed in adult rat cardiomyocytes. Stimulation of the α_1-adrenergic receptor by PE results in activation of Ca^{2+}-independent PKCs, as demonstrated by translocation of the δ and ε isoenzymes from cytosol to membrane fractions. PE also induces activation of p70 ribosomal protein S6 kinases (S6K1 and 2) in adult cardiomyocytes. We have studied the role of Ca^{2+}-independent PKCs in the regulation of S6K activity by PE. Activation of S6K1/2 by PE was blocked by the broad-spectrum PKC inhibitor bisindolylmaleimide (BIM) I, whereas Gö6976, a compound that only inhibits Ca^{2+}-dependent PKCs, did not inhibit S6K activation. Rottlerin, which selectively inhibits PKCδ, also prevented PE-induced S6K activation. The isoform-specific PKC inhibitors had similar effects on the phosphorylation of eukaryotic initiation factor 4E (eIF4E)-binding protein 1, a translation repressor that, like the S6Ks, lies downstream of the mammalian target of rapamycin (mTOR). Infection of cells with adenoviruses encoding dominant-negative PKCδ or ε inhibited the activation of extracellular-signal-regulated kinase (ERK) by PE, and also inhibited the activation and/or phosphorylation of S6Ks 1 and 2. The PE-induced activation of protein synthesis was abolished by BIM I and markedly attenuated by rottlerin. Our data thus suggest that Ca^{2+}-independent PKC isoforms play an important role in coupling the α_1-adrenergic receptor to mTOR signalling and protein synthesis in adult cardiomyocytes.

Key words: cardiac hypertrophy, phenylephrine, protein kinase C, protein synthesis, p70 S6 kinase, translation initiation.

INTRODUCTION

The protein kinase C (PKC) family, a group of at least 11 known members that possess phospholipid-dependent serine/threonine kinase activity, plays a key role in cellular signal transduction and is involved in the regulation of various cellular processes including neurotransmission, exocytosis, cell motility, contractility and cell growth [1,2]. PKC isoforms are expressed differentially and respond differently to physiological stimuli in diverse tissues and cell types [1–3]. The classical (Ca^{2+}-dependent) PKC (cPKC) α and the novel (Ca^{2+}-independent) PKC (nPKC) isoforms δ and ε have been detected immunologically in adult cardiomyocytes [3,4]. It is still controversial whether PKCβ isoforms are expressed in cardiomyocytes [5]. Although the identities of individual PKC isoforms have been known for some years, and distinct isoforms have been implicated in different cellular responses of cardiomyocytes such as hypertrophic growth, ischaemic preconditioning and apoptosis [6–9], the physiological substrates of these kinases and the downstream effects of individual PKC isoforms have nonetheless remained obscure.

The 70 kDa ribosomal protein S6 kinase 1 (p70 S6 kinase 1; S6K1) phosphorylates ribosomal protein S6 and is generally thought to be important in the increased translation of a subset of mRNAs characterized by the presence at their 5′-termini of a tract of pyrimidines ('5′-TOP mRNAs'; which encode ribosomal proteins and elongation factors) ([10], but see also [11]). In unstimulated cells, these mRNAs are poorly translated, but shift into polysomes upon stimulation. Thus activation of S6K1 is thought to up-regulate ribosome biosynthesis and increase the translational capacity of the cell. Activation of S6K1 involves its phosphorylation at multiple serine/threonine residues catalysed by several upstream kinases, and is dependent on signalling via the mammalian target of rapamycin (mTOR). Many studies, usually performed in cell lines in response to insulin or other growth factors, have demonstrated the necessity of the phosphoinositide 3-kinase (PI 3-kinase)/3-phosphoinositide-dependent protein kinase 1 (PDK1) and protein kinase B (also termed Akt) in the regulation of S6K1 [12,13] (probably via the hamartin/tuberin complex [14]). However, a small but growing body of data has suggested that other signalling events, including Ca^{2+}, PKC and mitogen-activated protein kinase (MAP kinase)/extracellular-signal-regulated kinase (ERK) kinase (MEK)/ERK signalling, are also important in the regulation of S6K1 activity [15–19]. With respect to PKC, some reports have suggested that atypical PKC isoforms are involved directly in the regulation of S6K1 activity as a component of PI 3-kinase signalling [20,21].

S6K2 is a recently identified homologue of S6K1 that phosphorylates S6 *in vitro* [22–25]. Several studies so far imply that S6K2 is regulated by PI 3-kinase signalling and by mTOR, similarly to S6K1 [24–27]. Like S6K1, S6K2 has been shown to

Abbreviations used: ARVC, adult rat ventricular cardiomyocytes; BIM, bisindolylmaleimide; eEF2, eukaryotic elongation factor 2; eIF4E, eukaryotic initiation factor 4E; 4E-BP1, eIF4E-binding protein 1; mTOR, mammalian target of rapamycin; PDK1, 3-phosphoinositide-dependent protein kinase 1; PE, phenylephrine; PI 3-kinase, phosphoinositide 3-kinase; PKC, protein kinase C; cPKC, classical PKC; nPKC, novel PKC; S6K, p70 ribosomal protein S6 kinase; ERK, extracellular-signal-regulated kinase; MAP kinase, mitogen-activated protein kinase; MEK, MAP kinase/ERK kinase; wt, wild-type; d/n, dominant-negative; GFP, green fluorescent protein; m.o.i., multiplicity of infection.

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be regulated by atypical PKCζ, which participates in PI 3-kinase signalling [26]. Our recent data indicate that MEK signalling is important for the activation of S6K2 by α_1-agonists in adult rat cardiomyocytes [27].

The α_1-adrenergic receptor agonist phenylephrine (PE), which acts via Gq-protein-coupled receptors, activates protein synthesis and exerts hypertrophic effects in cardiomyocytes [28–31]. Increased protein synthesis is a key feature of hypertrophy; and inhibition of protein synthesis could be a crucial step in apoptosis [31]. PE activates PKC and the MAP kinase cascade in cardiomyocytes [4,32–34] and these effects may be very important in mediating PE-induced cellular responses [30].

Our recent studies have shown that stimulation of protein synthesis by PE in adult rat ventricular cardiomyocytes (ARVC) involves activation or phosphorylation of targets of mTOR, such as S6K1/2 and the eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1), a repressor of cap-dependent translation [35]. Furthermore, regulation of S6K1/2 and 4E-BP1 by PE requires the activity of the classical MAP kinase pathway, the MEK/ERK cascade [19,27]. However, the signalling links between the α_1-adrenergic receptor and the regulation of protein synthesis still remain to be established fully. In view of the importance of PKC in cellular function and its involvement in α_1-adrenergic-receptor-linked cellular responses, and the critical role of protein synthesis in α_1-adrenergic-induced cardiac hypertrophy, we investigated the involvement of PKC isoforms in the regulation of S6Ks in adult cardiomyocytes. Here we demonstrate that regulation of S6Ks and 4E-BP1 and the activation of protein synthesis by α_1-receptor activation require Ca^{2+}-independent PKC isoforms.

MATERIALS AND METHODS

Materials

[γ-^{32}P]ATP, L-[^{35}S]methionine and ECL reagents were purchased from Amersham Biosciences. L-Microcystin, PD098059, rapamycin, bisindolylmaleimide (BIM) I, Gö6976 and rottlerin were from Calbiochem. BSA (fatty-acid-free) was from Boehringer Mannheim. Adult male Sprague–Dawley rats (250–300 g) were obtained from Charles River, Margate, Kent, U.K. All other chemicals or biochemicals were obtained from Sigma unless otherwise stated.

Isolation, culture, treatment and extraction of adult rat cardiomyocytes

Ventricular myocytes were isolated from hearts of adult rats, as described previously [36]. After isolation, ARVC were washed, seeded on to laminin-coated dishes and cultured as described previously [27,36]. Details of treatments are provided in the Figure legends: in all cases, controls received the appropriate vehicle. For studies in ERK activation, ARVC were treated with PE for only 5 min, by which time ERK activation is maximal [19,27]. Phosphorylation of S6Ks and 4E-BP1 is considerably slower, and to study the control of these proteins cells were therefore treated for longer periods (usually 1 h). Cell lysates were prepared either as described previously [36] or as detailed under 'Preparation of cytosol and membrane fractions'. Protein concentrations were determined by the Bradford method [37].

Adenoviral-mediated gene transfer

The recombinant adenovirus vectors carrying wild-type (wt) PKCδ (wt PKCδ) and dominant-negative (d/n) PKCδ (K376A; d/n PKC) were provided kindly by Professor T. Kuroki (Showa University, Tokyo, Japan) [38,39]. Recombinant adenovirus vectors carrying wt PKCε and d/n PKCε were also generous gifts from Professor T. Kuroki (K436R mutant of the rabbit protein) [40] and Dr C. Schmitz-Peiffer (Garvan Institute, Sydney, Australia; equivalent K437R mutant of the rat protein) [41], who also provided the adenoviral green fluorescent protein (GFP) vector. After isolation ARVC were cultured in 60-mm plates for 2 h before the infection was carried out. ARVC cultures were washed and incubated in 1 ml of M199 medium containing recombinant adenoviruses for 2–3 h at 37 °C at the indicated multiplicities of infection (m.o.i.) given in the Figure legends. Cultures were given fresh M199 medium and incubated for further 36 h before subsequent treatments. To perform mock infection as a control and to assess the efficiency of infection, ARVC were infected with adenovirus containing LacZ or GFP. The infectivity of the adenovirus was greater than 95 % based upon β-galactosidase staining of cells or by inspection under a fluorescence microscope.

Preparation of cytosol and membrane fractions

Cultured ARVC were washed in cold PBS without Ca^{2+} or Mg^{2+} and harvested in buffer A containing 20 mM Tris, pH 7.5, 2.5 mM EGTA, 1.0 mM EDTA, 100 mM NaF, 2 μg/ml pepstatin, 2 mM dithiothreitol and 1 mM PMSF. The suspension was sonicated for 20 s and then centrifuged at 1500 ***g*** for 10 min. Supernatant was collected and centrifuged at 100 000 ***g*** for 45 min at 4 °C, the cytosol supernatant fraction was retained, and the pellet was solubilized in buffer A containing 1 % Triton X-100 and sonicated. The suspension was centrifuged at 15 000 ***g*** for 15 min at 4 °C. The resulting supernatant was collected as the membrane fraction. Equal amounts of protein were resolved by SDS/PAGE for subsequent immunoblot analysis.

SDS/PAGE and immunoblotting

SDS/PAGE and Western blotting were performed as described previously [42,43]. The anti-S6K2 antibody was prepared as described previously [22]. Anti-(phospho-ERK1/2) antibody was supplied by New England Biolabs. Anti-ERK2 antibody was from Transduction Laboratories. Anti-PKCα, anti-PKCδ and anti-PKCε antibodies were obtained from Santa Cruz Biotechnology. Western blots of S6K1, eIF4E and 4E-BP1, eukaryotic elongation factor 2 (eEF2) and phospho-eEF2 were performed as described previously [19,27,36].

m^{7}GTP–Sepharose chromatography

To assess the interaction of eIF4E with 4E-BP1, we performed m^{7}GTP–Sepharose chromatography [43]. Briefly, 20 μl of a 50/50 slurry of m^{7}GTP–Sepharose CL-4B was mixed with 1.0 mg of ARVC lysate protein. The mixtures were rotated for 1 h at 4 °C, and the m^{7}GTP–Sepharose and associated proteins were pelleted by centrifugation. The beads were then washed three times in extraction buffer (buffer A). For SDS/PAGE, proteins bound to m^{7}GTP–Sepharose were removed from the matrix by boiling in SDS sample buffer.

In vitro kinase assays

The activity of PKCδ was assayed using myelin basic protein as a substrate after immunoprecipitating the cell lysates with anti-PKCδ antibody. Briefly, treated/infected or non-treated/uninfected cells were washed in cold PBS without Ca^{2+} and Mg^{2+} and harvested in extraction buffer (buffer A). Immunoprecipitates

were used for kinase assay in the kinase reaction buffer (20 mM Tris/HCl, pH 7.5, 25 mM β-glycerophosphate, 1 mM Na_3VO_4 and 1 mM dithiothreitol) containing 33 μg/ml myelin basic protein, 0.5 mM phosphatidylserine (which was sonicated three times for 1 min on ice just before use), 1 μM PMA, 2 mM $MgCl_2$/100 μM ATP and 2 μCi of [γ-^{32}P]ATP/assay in a total volume of 30 μl. PKC activity was corrected by subtraction of non-specific incorporation determined by assay without myelin basic protein and expressed as the percentage of the value from control cells.

The activities of S6K1 and S6K2 were assayed using a specific peptide substrate after immunoprecipitation, as described previously [27,36].

Measurement of protein synthesis

Cultured ARVC were preincubated with fresh M199 with or without PKC inhibitors. Cells were then stimulated with PE for 1.5 h before the addition of L-[^{35}S]methionine (10 μCi/ml) for a further 30 min. For adenovirus-infected cells, fresh M199 was added 36 h after infection and the cells were then either incubated with L-[^{35}S]methionine (10 μCi/ml) for a further 30 min or stimulated with PE for 1.5 h before the addition of L-[^{35}S]methionine (as indicated in the Results section). Cells were washed three times with ice-cold PBS and were then lysed with extraction buffer (buffer A). Proteins were collected by filtration on 3MM filters (Whatman) before precipitation with 10 % (w/v) trichloroacetic acid and measurement of incorporated radiolabel by scintillation counting.

RESULTS

PE activates Ca^{2+}-independent PKC isoforms, and not Ca^{2+}-dependent PKCs in ARVC

Activated PKC is associated with membrane fractions, due to its binding to lipid. Translocation studies were therefore performed to examine whether PKC was activated by PE under the experimental conditions we applied and to test which isoforms were activated. Exposure of cells to PE caused a sustained increase in the levels of the Ca^{2+}-independent PKC (nPKC) isoforms δ and ε found in the membrane fraction after cell fractionation, as shown by Western blotting (Figure 1A). The apparent decrease in the levels of PKCε in the cytosol was not as obvious as the increase in the membrane-associated fraction, but cytosolic amounts of PKCδ were clearly decreased (Figure 1A). The translocation of the δ-subtype did not occur as rapidly as reported previously [4,34] for neonatal cardiomyocytes; instead, it was apparent only after 2.5 min stimulation. Furthermore, both PKCδ and PKCε were retained in the membrane fraction at least up to 30 min after stimulation of the cells, although some relocalization of PKCε to the cytoplasm was evident by 15 min (Figure 1A). Translocation of the Ca^{2+}-dependent cPKC isoform α was not observed upon PE treatment, indicating that PE does not activate PKCα in this cell type (Figure 1B). As a positive control, cells were treated with the phorbol ester PMA, which did induce a marked translocation of PKCα from the cytosol to the membrane (Figure 1B). These results are consistent with previous reports showing that PE activates nPKCδ and nPKCε but not cPKCα in adult cardiomyocytes [4].

PE-induced ERK activation requires Ca^{2+}-independent PKC activities

PE was reported previously to elicit a rapid and sustained activation of both ERK1 and ERK2 in ARVC [19,27]. Our data show that the activation of ERK1/2 by PE was reduced significantly in the presence of the broad-spectrum PKC inhibitor BIM I, which inhibits both Ca^{2+}-dependent and Ca^{2+}-independent PKC isoforms [44,45] (Figure 2A).

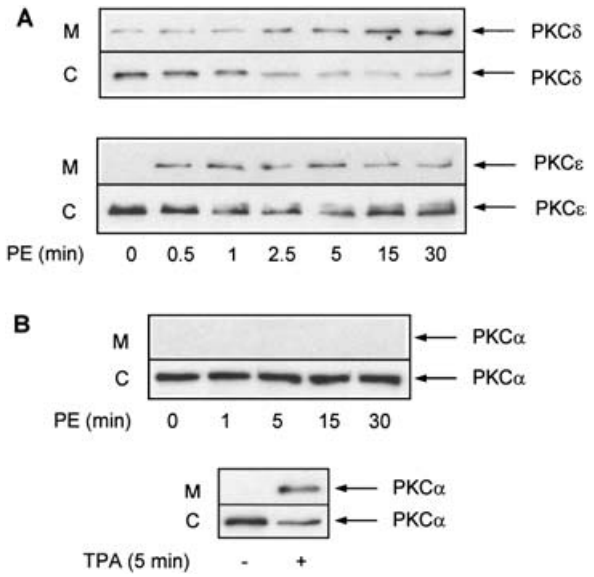

Figure 1 PE induces translocation of Ca^{2+}-independent PKCδ and PKCε but not Ca^{2+}-dependent PKCα in adult cardiomyocytes

Cultured ARVC were treated with PE (10 μM) for the times indicated (**A**, and top panel of **B**) or PMA (TPA; 20 nM) for 5 min (bottom panel of **B**). Cells were harvested and cytosol (C) and membrane (M) fractions were prepared as described in the Materials and methods section. Protein concentrations were determined and equal amounts of total protein (20–40 μg) were used for Western blotting analysis using isoform-specific PKC antibodies. Data are representative of five experiments performed.

Rottlerin is a selective inhibitor of PKCδ, with an IC_{50} *in vitro* of 3–6 μM [46]. Rottlerin also inhibits eEF2 kinase *in vitro*, with its IC_{50} being about 5 μM [47], i.e. similar to the concentration at which it inhibits PKCδ. In our hands, rottlerin was detrimental to ARVC when used at concentrations in this range, whereas no loss of cell viability was observed at lower concentrations, e.g. 0.5 μM, the concentration used here. However, this concentration would appear to be too low to inhibit targets such as PKCδ and eEF2 kinase. Nonetheless, treatment of ARVC with rottlerin consistently led to a marked dephosphorylation of eEF2 (Figure 2B), presumably due to inhibition of eEF2 kinase. This suggests that either rottlerin is a much more potent inhibitor *in vivo* than *in vitro*, or, perhaps more likely, that its effective concentration within the cells is higher than its extracellular concentration (for example, cells may actively take up this compound).

Rottlerin also almost completely inhibited the activation of ERK induced by PE (Figure 2A). On the other hand, pretreatment of the cells with Gö6976 [48,49], an inhibitor of Ca^{2+}-dependent PKCs, had no obvious effect on ERK activation (Figure 2A). However, Gö6976 did inhibit the activation of ERK by PMA, which potently activates Ca^{2+}-dependent PKC isoforms (Figures 1B and 2C), thus confirming the efficacy of this compound at the concentrations used in this study. These results suggest that activation of ERK1/2 by PE in ARVC, like that in neonatal cells [34], requires Ca^{2+}-independent PKCs but not Ca^{2+}-dependent PKC isoforms. Since rottlerin is a relatively poor inhibitor of PKCε (*in vitro* IC_{50} = 80–100 μM [46]), it is possible that the effect of PE is mediated primarily via PKCδ.

To obtain more evidence for the possible involvement of Ca^{2+}-independent PKCs in the activation of ERK by PE, we infected

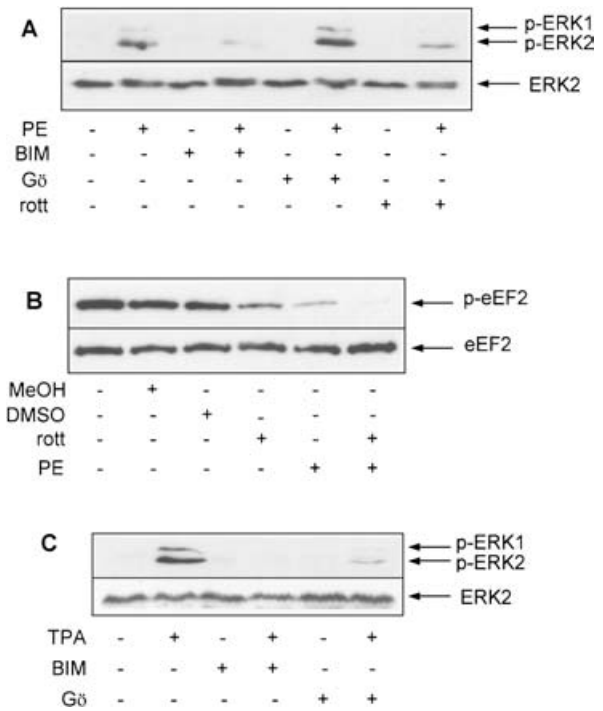

Figure 2 The role of Ca^{2+}-independent PKCs in the phosphorylation (activation) of ERK1/2 in response to PE

(**A**) Cultured ARVC were incubated with BIM I (5 μM), Gö6976 (Gö; 2 μM) or rottlerin (rott; 500 nM) for 30 min as indicated prior to the addition of PE (10 μM) for 5 min. Activation of ERK1/2 was assessed by Western-blotting analysis using a phospho-specific antibody that recognizes ERK1/2 phosphorylated at Thr-202/Tyr-204 (p-ERK1/2; upper blot in each panel). An antibody that detects ERK2 irrespective of its phosphorylation state was used to verify equal loading of the samples (ERK2; lower immunoblot in each panel). Data are representative of three experiments performed. (**B**) ARVC were treated with PE (10 μM) for 60 min, where indicated, in the presence or absence of rottlerin (500 nM, 30 min). Samples were analysed by SDS/PAGE and Western blotting using antiserum for phosphorylated eEF2 (Thr-56; p-eEF2; upper blot). Parallel blots were performed using an antibody that recognizes eEF2 irrespective of its state of phosphorylation to verify equal loading for all treatments (lower blot). Data are representative of two experiments performed. MeOH, methanol. (**C**) As for (**A**), but ARVC were treated with PMA (TPA; 20 nM) for 5 min. Data are representative of three experiments performed.

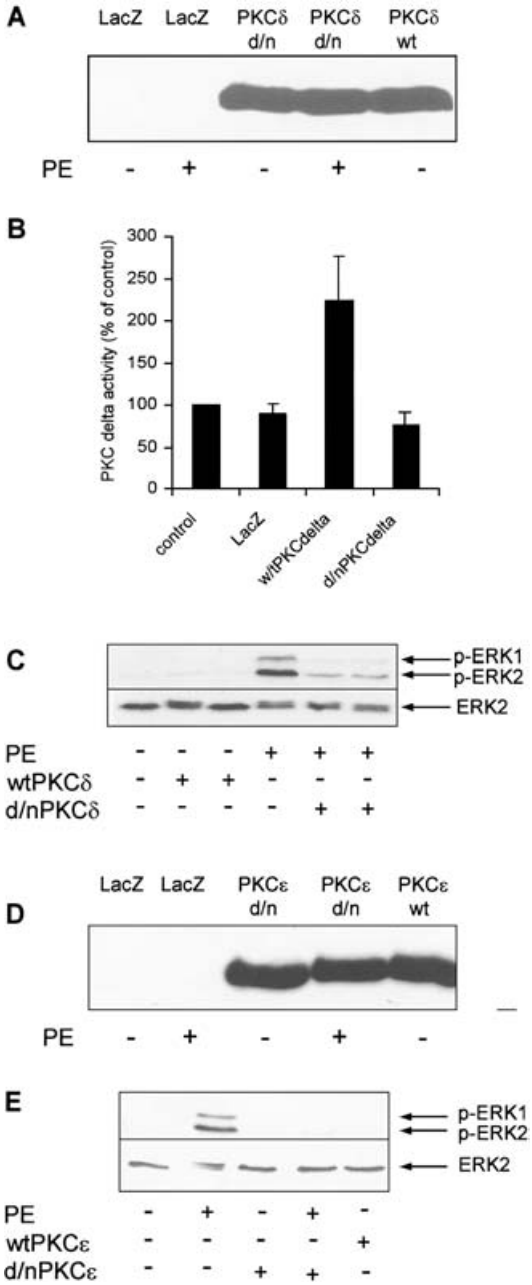

Figure 3 Dominant-inhibitory mutants of PKCδ or ε interfere with PE-induced ERK activation

ARVC were infected, where indicated, with adenoviruses encoding LacZ (LacZ, virus infection control), wt PKCδ or d/n PKCδ at an m.o.i. of 50 p.f.u./cell for 36 h (**A**–**C**) or encoding GFP, wt PKCε or d/n PKCε **(D**, **E)** as described in the Materials and methods section. Control indicates uninfected cells. Where indicated, cells were treated with PE (10 μM) for 5 min and were then lysed. Samples of lysate were either processed for Western blotting for PKCδ (**A**) or PKCε (**D**) or determination of PKCδ activity (**B**). In (**B**), data are presented as a percentage of the uninfected control, set at 100 % (means $\pm$ S.D., $n = 4$); statistical comparison of wt PKCδ versus control showed that the difference was significant ($P < 0.01$). (**C**, **E**) Samples of lysate were also analysed by SDS/PAGE/immunoblotting for ERK activation, as in Figure 2.

ARVC with adenoviral vectors encoding wt or d/n PKCδ or PKCε. Infection of ARVC with increasing amounts (m.o.i.) of the adenoviruses encoding PKCδ led to increasing levels of expression of the PKCδ protein, detected using a specific antibody (results not shown). Levels of PKCδ expression in a typical experiment are shown in Figure 3(A).

Expression of wt PKCδ caused a substantial increase in PKCδ activity measured in the total cell lysates (Figure 3B), but failed to elicit activation (phosphorylation) of ERK1/2 (Figure 3C). Thus overexpression of wt PKCδ alone does not activate the ERKs and additional regulatory events therefore appear to be required for this. Expression of d/n PKCδ slightly reduced the basal PKCδ activity in ARVC (Figure 3B) and almost completely inhibited the activation of ERK by PE (Figure 3C).

Adenovirus-mediated expression of a dominant-interfering form of PKCε also blocked the activation of ERK by PE, while expression of a wild-type PKCε mutant failed to activate ERK (Figures 3D and 3E). The d/n PKCε mutant also consistently blocked ERK activation at 3 min after PE addition (results not shown). These data provide a very similar picture to that observed for PKCδ and suggest that the activation of MEK/ERK signalling requires the activity of the Ca^{2+}-independent PKC isoforms δ and ε. Similar data were obtained with the two different sets of PKCε vectors employed that encode wt or dominant-interfering mutants of rabbit or rat PKCε (see the Materials and methods section).

Ca^{2+}-independent PKCs are involved in the activation of S6K1/2 and phosphorylation of 4E-BP1 by PE

The above data indicate that Ca^{2+}-independent PKC isoforms are involved in ERK activation in ARVC. We have shown recently that the MEK/ERK pathway is required for the stimulation of S6K1 and S6K2 by PE [19,27]. Therefore, we tested the role of nPKC isoforms in the activation of S6Ks in response to α_1-receptor stimulation. Both S6K1 and S6K2 are activated by PE, with maximal activity of both kinases being reached at 60 min after PE treatment, after a short delay [19,27]. Activation of S6K1 by PE was blocked completely by the selective α_1-antagonist prazosin, indicating that this effect is indeed mediated via the α_1-adrenergic receptor (results not shown).

BIM I pretreatment almost completely prevented the activation of S6K1/2, while the cPKC isoform-specific inhibitor Gö6976 had no apparent effect (Figure 4A). To eliminate possible effects due to direct inhibition by BIM I of S6K activity [50], S6K kinase assays were carried out on S6K immunoprecipitates which had been washed thoroughly to ensure the kinase reaction mixtures were free of BIM I. BIM I does not affect the activity of S6K1 in immunoprecipitates from untreated control cells (Figure 4A), confirming the lack of interference of BIM I with the S6K assay. The basal activities of both S6K1 and S6K2 were significantly reduced by pretreatment of cells with rottlerin, and S6K activation was abolished by this compound (Figures 4A and 4B). These data suggest that Ca^{2+}-independent PKCs are involved in S6K activation. As ERK activation by PE also requires Ca^{2+}-independent PKC isoforms (Figure 3), these results indicate that Ca^{2+}-independent PKCs may act upstream of MEK/ERK and mediate PE signalling to S6Ks. We cannot, of course, rule out the possibility that some isoforms of PKC can also act in parallel to MEK/ERK in signalling from PE to the S6Ks (see also Discussion).

PE also induces the phosphorylation of 4E-BP1 in ARVC [19]. This allows it to be released from eIF4E leading to increased formation of the eIF4F translation factor complex [19], which is important for recruitment of ribosomes to the mRNA and for efficient initiation of normal cap-dependent translation [35]. This effect of PE should thus lead to enhanced initiation of translation. As the regulation of 4E-BP1 by PE in ARVC is linked, like that of S6K1 and S6K2, to mTOR and ERK [19,27], the role of PKC in PE-induced 4E-BP1 phosphorylation was also tested. Phosphorylation of 4E-BP1 is judged by a shift in its migration on SDS/PAGE, where three species can be resolved, the fastest migrating α being the least phosphorylated, while the slowest γ form is the most highly phosphorylated one. In unstimulated cells, 4E-BP1 was present as the α- and β-forms. PE induced a shift in the mobility of 4E-BP1 on SDS/PAGE towards slower-migrating, more heavily phosphorylated forms (β and γ; Figure 4C). Consistent with the effects of the PKC inhibitors on S6K activation, pretreatment of ARVC with BIM I or rottlerin blocked the ability of PE to elicit increased phosphorylation of 4E-BP1, while inhibition of Ca^{2+}-dependent PKC activity by preincubation of cells with Gö6976 had no effect (Figure 4B). Hyperphosphorylation of 4E-BP1 prevents it from binding to eIF4E (reviewed in [35]) and, as expected, the PE-induced phosphorylation of 4E-BP1 led to its release from eIF4E, as assessed by Western blotting of proteins bound to m^7GTP–Sepharose, a resin which specifically binds eIF4E and thus any proteins associated with eIF4E (Figure 4D). This effect was abrogated by pretreatment of ARVC with BIM I or rottlerin, but not by Gö6976 (Figure 4D), consistent with the above data. Thus PE-induced phosphorylation of 4E-BP1 and its subsequent release from eIF4E, like the activation of S6K1/2, also require Ca^{2+}-independent PKC activities.

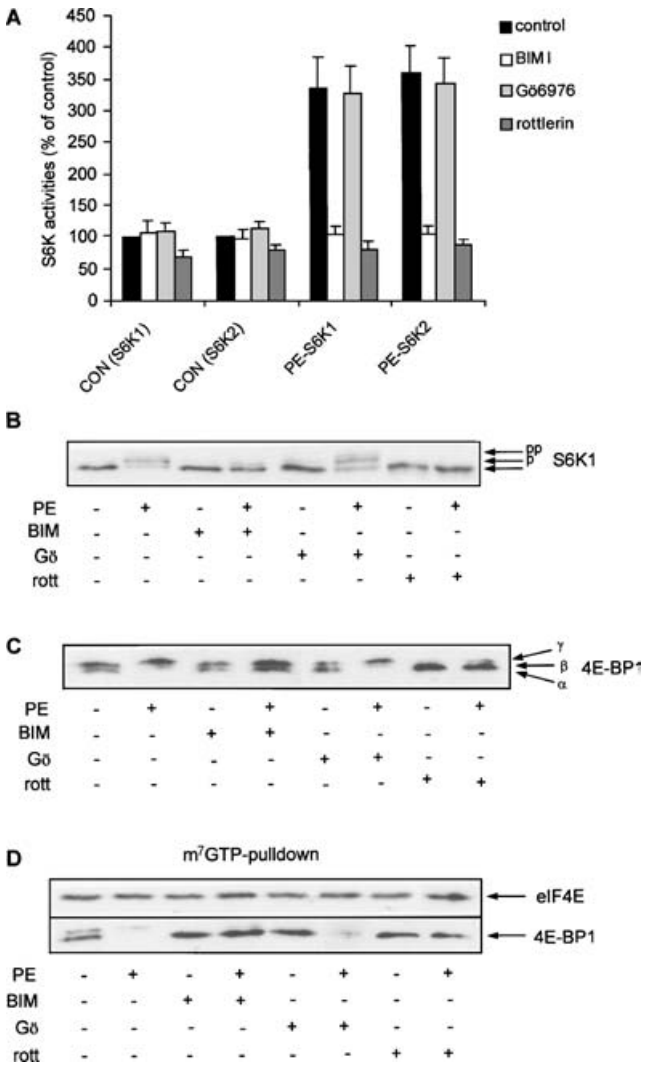

Figure 4 Ca^{2+}-independent PKC activities are required for the activation of S6K1/2 and phosphorylation of 4E-BP1 by PE in ARVC

(**A**) and (**B**) Cultured ARVC were pretreated, as indicated, with BIM I (5 μM), Gö6976 (Gö; 2 μM) or rottlerin (rott; 500 nM) for 30 min prior to the addition of PE (10 μM, 1 h). Cells were then harvested and extracts were processed for measurement of S6K1 and S6K2 activity (see the Materials and methods section). Assays were performed in duplicate and the data are derived from four separate experiments using different batches of ARVC [data are means $\pm$ S.D. expressed relative to the activity in unstimulated/untreated control cells (CON)] (**A**). Cell extracts from the same treatments were also subjected to SDS/PAGE and immunoblotting using anti-S6K1 antiserum as described in the Materials and methods section (**B**). Labelled arrows indicate the positions of differentially phosphorylated species of S6K1, 'pp' denoting the most highly phosphorylated form. (**C**) ARVC were treated as in (**A**) and (**B**) and samples of cell lysate were analysed by SDS/PAGE and Western blotting using an anti-4E-BP1 antibody. The gel system used resolves three species of 4E-BP1, α–γ, γ being the most highly phosphorylated (positions are indicated). (**D**) Samples of cell lysate (1000 μg) from cells subjected to the same treatment as in (**C**) were subjected to affinity chromatography on m^7GTP–Sepharose and the bound proteins were analysed by SDS/PAGE and Western blotting using antisera to eIF4E and 4E-BP1. Data in (**B**)–(**D**) are representative of four experiments, in which essentially identical results were obtained.

Doubts have been expressed about the specificity of BIM I and rottlerin for PKCs [50,51] (although there is no evidence that eEF2 kinase, which is affected by rottlerin, is involved in their control). We therefore considered it important to employ an additional and independent approach to examine the involvement of Ca^{2+}-independent PKCs in the regulation of S6K1/2 and

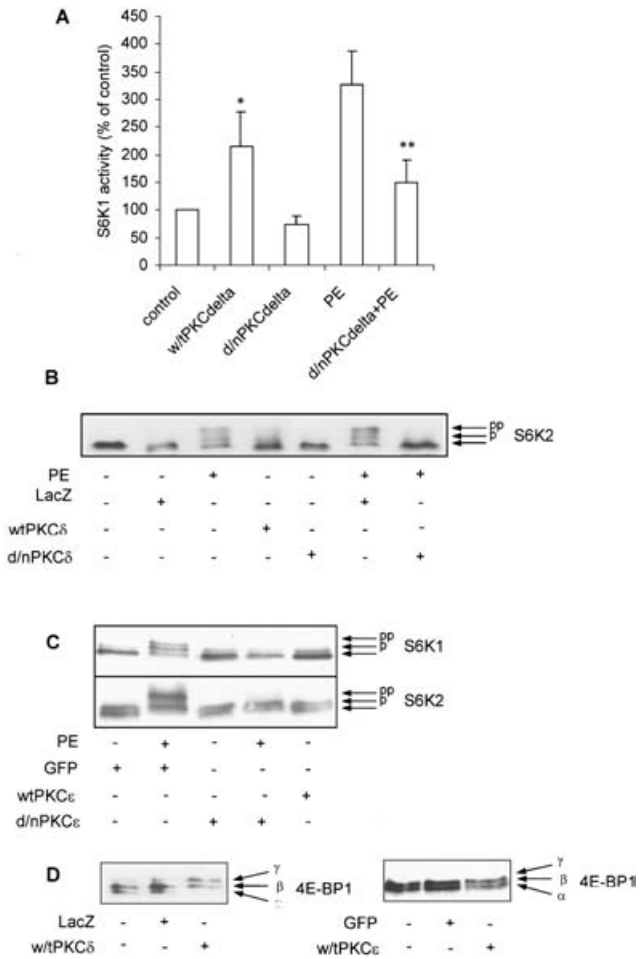

Figure 5 Use of adenovirus infection approaches to study the involvement of the Ca^{2+}-independent isoform PKCδ in the regulation of S6Ks and 4E-BP1

(**A**) and (**B**) ARVC were infected with adenoviruses encoding LacZ (control), wt PKCδ or d/n PKCδ at the m.o.i. of 50 p.f.u./cell. Cells were treated with PE, where indicated, and were extracted. Samples of lysate were assessed for S6K1 activity (**A**): in this case, data are presented as a percentage of AdLacZ-infected control, set at 100 % (means $\pm$ S.D., $n = 4$; $^{*}P < 0.01$ versus control, $^{**}P < 0.01$ versus PE). Cell lysates were also subjected to SDS/PAGE and Western blotting using anti-S6K2 antiserum as described in the Materials and methods section (**B**). Labelled arrows indicate the positions of differentially phosphorylated species of S6K2, 'pp' denoting the most highly phosphorylated form. (**C**) As for (**B**), but cells were infected with adenoviruses encoding GFP, wtPKCε or d/nPKCε as indicated and treated with PE as shown. Samples of cell lysate were analysed by SDS/PAGE and Western blotting to assess the states of phosphorylation of S6K1 (upper section) or S6K2 (lower section), with annotation as for (**B**). (**D**) Cultured ARVC were infected with adenoviruses expressing either β-galactosidase (LacZ) or wt PKCδ at the same m.o.i. (50 p.f.u./cell), or with adenoviruses encoding GFP or wt PKCε, as indicated. The cell lysates were used for Western blots for 4E-BP1 as in Figure 3(C). Results in (**B**)–(**D**) are representative of three Western blots performed.

4E-BP1. ARVC were therefore infected with adenoviruses expressing wt or d/n variants of PKCδ/ε. As shown above, PKCδ activity was elevated in cells infected with wt PKCδ adenovirus (Figure 3B). Although expression of wt PKCδ did not cause activation of ERK (Figure 3C), infection of ARVC with this virus still brought about a significant increase in S6K1 activity (Figure 5A) or S6K2 phosphorylation (assessed by electrophoretic mobility, indicative of activation [27]; Figure 5B). The PE-induced activation of S6K1 and S6K2 was reduced significantly in cells infected with d/n PKCδ compared with that in mock-infected cells (Figures 5A and 5B). The incomplete nature of the inhibition observed may reflect the incomplete inhibition of ERK

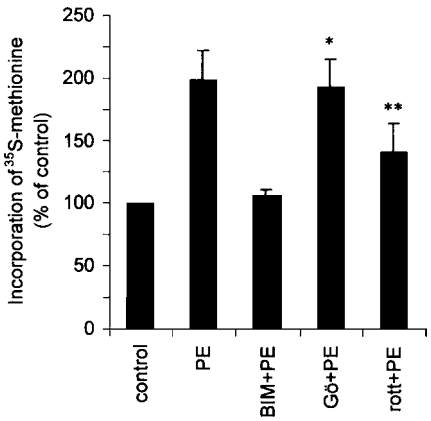

Figure 6 Activation of protein synthesis by PE is blocked by inhibition of Ca^{2+}-independent PKC activity

ARVC were cultured overnight and then treated with PE (10 μM) for 1.5 h, where indicated. In some cases, cells were preincubated with BIM I (5 μM for 30 min), Gö6976 (Gö; 2 μM for 30 min) or rottlerin (rott; 500 nM for 30 min) prior to addition of agonists. Protein synthesis was assessed by measuring incorporation of [^{35}S]methionine into protein as described in the Materials and methods section. Data are presented as percentages of the unstimulated control, set at 100 % (means $\pm$ S.D., $n = 5$). The percentage of stimulation by PE in the presence of inhibitors was corrected for any effect of the inhibitors on the level of protein synthesis in untreated controls, where appropriate. $^{*}P > 0.5$ versus PE (non-significant), $^{**}P < 0.02$ versus PE.

activation by this PKCδ mutant (Figure 3C) or effects of PE mediated via other nPKC isoforms, such as PKCε (Figure 1A).

Expression of a dominant interfering form of PKCε also blocked the PE-induced phosphorylation of S6K1 or S6K2, as judged by their mobilities on SDS/PAGE (Figure 5C). Expression of the wild-type form of PKCε did not itself elicit any significant shift in the mobility of S6K1 or S6K2, which differs from the situation for PKCδ, which did appear to elicit a modest activation or increase in the phosphorylation of these enzymes.

Adenovirus infection at an m.o.i. of 50 p.f.u./cell can itself induce 4E-BP1 phosphorylation ([52]; LacZ control in Figure 5D), but phosphorylation of 4E-BP1 was still further increased in cells overexpressing wt PKCδ compared with control cells expressing β-galactosidase infected at the same m.o.i. (Figure 5D). This result thus indicates again the important role PKCδ plays in the regulation of 4E-BP1 phosphorylation. In contrast, adenovirus-mediated expression of PKCε had no effect on the level of phosphorylation of 4E-BP1 (Figure 5D).

Activation of protein synthesis by PE requires Ca^{2+}-independent PKC activity

As regulation of S6K1/2 and 4E-BP1 plays an important role in the control of mRNA translation initiation, we studied whether the treatment of ARVC with PKC inhibitors affected the regulation of protein synthesis by α_1-receptor activation. Treatment of PE led to a substantial activation of the rate of overall protein synthesis, as measured by the acute incorporation of [^{35}S]methionine into newly synthesized protein (Figure 6). The stimulation of protein synthesis by PE was blocked by pretreatment of ARVC with BIM I. Rottlerin also partially inhibited the incorporation of label. It was possible that the smaller inhibition by rottlerin of PE-activated protein synthesis relative to its effect on S6K activation and 4E-BP1 phosphorylation reflected the inhibitory effect of this compound on eEF2 kinase which leads to decreased phosphorylation, and thus activation, of eEF2 in ARVC (see Figure 2B [47]; see also the Discussion). The smaller effect of

rottlerin may thus reflect two opposing actions of this compound – on the one hand, it blocks the activation of S6K and the phosphorylation of 4E-BP1, which would be expected to impair the activation of protein synthesis, while on the other hand, it leads to the dephosphorylation and activation of eEF2 which would have the opposite effect.

When corrected for the fact that it suppressed the rate of protein synthesis in control cells (results not shown; for details see legend to Figure 6), Gö6976 had little if any effect on the ability of PE to activate protein synthesis in ARVC (Figure 6). Thus inhibition of Ca^{2+}-independent nPKC activity, but not cPKCs, impairs the activation of overall protein synthesis by PE, consistent with the fact that PE-induced Ca^{2+}-independent PKC activation is involved in the regulation of the translational regulators S6K1/2 and 4E-BP1.

DISCUSSION

The data presented here show for the first time that stimulation of translation regulators linked to mTOR, such as S6K1/2 and 4E-BP1, and the activation of protein synthesis by the α_1-adrenergic agonist PE in adult cardiomyocytes requires the activities of Ca^{2+}-independent PKCs. The ability of PE to elicit phosphorylation of these proteins is blocked by inhibitors of Ca^{2+}-independent PKC isoforms and also by overexpression of dominant-inhibitory forms of PKCδ or PKCε. Interfering with PKCδ or PKCε (using inhibitors or by expressing d/n mutants) also blocks the activation of ERK. Both isoforms are activated by PE, but with slightly different kinetics, so the relative contribution of each to downstream signalling may vary according to the time after addition of agonist.

The ability of BIM I, but not Gö6976, to block the effects of PE on S6K1/2 and 4E-BP1 indicates an essential role for Ca^{2+}-independent nPKCs, rather than cPKCs, in these signalling events. Since BIM I (but not Gö6976) also blocked ERK activation, Ca^{2+}-independent PKCs appear to play a role in the upstream, receptor-proximal, signalling events coupled to the α_1-adrenergic receptor, which include activation of the Ras/MEK/ERK signalling pathway (Figure 2; [19,27]). Our studies using infection of ARVC with adenoviruses encoding dominant-inhibitory (kinase-dead) forms of PKCδ/ε also support this conclusion. The expression of dominant-interfering mutants of PKCδ or ε blocked PE-induced ERK activation and phosphorylation of S6K1 and S6K2. Thus Ca^{2+}-independent PKCs appear to affect the regulation of S6Ks and 4E-BP1 via Ras/ERK signalling.

Since rottlerin, which inhibits PKCδ much more potently than PKCε [46], blocks the activation/phosphorylation of S6K1/2 and the phosphorylation of 4E-BP1, it is possible that PKCδ plays a more important role than PKCε in upstream signalling from the α1-adrenergic receptor to these targets of the mTOR pathway. Furthermore, there was a hint that overexpression of PKCδ but not PKCε led to activation of the S6Ks and to increased phosphorylation of 4E-BP1. It thus seems possible that PKCδ plays a more important role than PKCε in their regulation, possibly by additional signalling links other than the control of the ERKs.

Our data suggest that both PKCδ and PKCε are involved in the activation of ERKs by PE in ARVC. Alternatively, since it seems likely that dominant-interfering mutants 'work' by inhibiting or sequestering upstream activators of the wt enzyme, it may well be that a dominant-interfering mutant of PKCε may also impair activation of PKCδ, and *vice versa*, since these isoforms are very similar. For example, they probably require the same ligands for activation, and, since they contain very similar sites of phosphorylation, are likely substrates for similar, or identical, upstream kinases. For technical reasons, it is extremely hard to assess whether a dominant-interfering form of PKCε blocks the regulation of PKCδ. Despite numerous attempts, it proved impossible to see activation of PKCδ in ARVC in response to PE using standard assays (L. Wang and C. G. Proud, unpublished work). We also tried to examine whether PE or interfering PKC mutants affected the phosphorylation state of the endogenous PKCδ/ε using commercially available phosphospecific antisera. Again, this proved impossible, due in this case to the very low levels of expression of PKC isoforms in ARVC, which meant that no signal was observed in our Western blots.

The work of Heidkamp et al. [7] provided evidence supporting the notion that PKCε, rather than PKCδ, is involved in the activation of ERK. This differs in part from our own conclusions, which suggest a role for PKCδ and possibly also for PKCε in the activation of this pathway. There are two important differences between their experiments and ours. Firstly, Heidkamp et al. [7] used a constitutively active form of PKCε, whereas our data were obtained using wt or d/n forms. Secondly, and probably more importantly, their studies used neonatal rat ventricular myocytes, while we have used myocytes from adult animals. There are a number of differences in terms of signalling connections between neonatal and adult rat cardiomyocytes, and this may well explain the difference between our findings. For example, PE activates c-Jun N-terminal kinase in neonatal cardiomyocytes [53], but not in the adult cells that we use [27].

It is also possible that certain PKC isoforms are involved at relatively downstream stages in the signalling processes which regulate translation factors; recent data from Parekh et al. [54] suggested a possible role for the mTOR pathway in the phosphorylation and activation of PKCδ and PKCε, and the study by Kumar et al. [55] indicated a role for PKCδ in the mTOR-linked signalling events that control the phosphorylation of 4E-BP1. In this study, we show that inhibition of PKCδ by rottlerin completely blocked the effects of PE on S6Ks and 4E-BP1, whereas activation of ERK was only partially affected. On the other hand, as mentioned above, although wt PKCδ expression did not cause any apparent change in ERK activation, it did elicit an increase in S6K activity. These observations probably imply that PKCδ functions both upstream of and in parallel to ERK in PE signalling to the regulation of S6Ks and 4E-BP1. To study whether there is a direct involvement of PKCδ in mTOR signalling in ARVC, we also examined PKCδ translocation in the presence of the mTOR inhibitor rapamycin. Rapamycin sometimes reversed the relocalization of PKCδ induced by PE (results not shown). However, this result was not very reproducible, and we have not addressed the issue further. Therefore, the present data do not provide information to tell us whether PKCδ (and PKCε) act parallel to mTOR or downstream of it, as well as upstream of ERK.

Rottlerin has been reported to inhibit eEF2 kinase with a potency similar to that for its effect on PKCδ [47]. eEF2 kinase phosphorylates eEF2, which in turn inhibits peptide-chain elongation. Thus inhibition of eEF2 kinase results in an increase in protein synthesis [56]. Our results indeed demonstrated that rottlerin caused dramatic dephosphorylation of eEF2 in ARVC: it not only reduced eEF2 phosphorylation in control cells, but also augmented the PE-induced dephosphorylation of eEF2, which also contributes to the activation of protein synthesis by PE. (Its ability to inhibit PE-induced activation of the upstream regulators of eEF2 kinase, S6K1 and the ERK pathway [56], would lead to the opposite effect, so the inhibitory effect of rottlerin on eEF2 kinase is clearly the dominant one here). The compound therefore has two, mutually antagonistic, effects on the protein synthetic machinery in this system. This may explain, at least in

part, the observation that inhibition of PKCδ by rottlerin completely abolished S6K activation and 4E-BP1 phosphorylation by PE, whereas the PE-induced increase in protein synthesis was only partially inhibited.

These effects underlie, or at least contribute to, the ability of PE to activate protein synthesis in these cells. Clearly, this may be very important in the hypertrophic effect of PE in cardiac myocytes that involves an increase in the protein content of the cells. As Ca^{2+} levels in cardiac cells fluctuate markedly on a beat-to-beat basis, signalling via nPKC isoforms that are insensitive to changes in Ca^{2+} concentration would be a reasonable way for agonists to regulate cell functions in this cell type.

In summary, the present study provides new evidence for the involvement of Ca^{2+}-independent PKC isoforms in the stimulation of mRNA translation and components of the translational machinery in adult cardiomyocytes in response to α_1-receptor activation. Using two different approaches we have provided evidence that the activation of ERK in response to PE requires Ca^{2+}-independent PKC isoforms. Furthermore our data indicate that the hypertrophic agent PE induces increased S6K activity and 4E-BP1 phosphorylation via a pathway which not only involves Ras/ERK, but also requires Ca^{2+}-independent PKCs. Whether PKCδ (and ε) are directly involved in the regulation (phosphorylation) of S6Ks and 4E-BP1 remains to be established.

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