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Cellular Signalling 18 (2006) 225 – 235

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# Peptide growth factors signal differentially through protein kinase C to extracellular signal-regulated kinases in neonatal cardiomyocytes

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Received 8 March 2005; received in revised form 15 April 2005; accepted 19 April 2005
Available online 3 June 2005

**Abstract**

The extracellular signal-regulated kinases 1/2 (ERK1/2) are activated in cardiomyocytes by Gq protein-coupled receptors and are associated with induction of hypertrophy. Here, we demonstrate that, in primary cardiomyocyte cultures, ERK1/2 were also significantly activated by platelet-derived growth factor (PDGF), epidermal growth factor (EGF) or fibroblast growth factor (FGF), but insulin, insulin-like growth factor 1 (IGF-1) and nerve growth factor (NGF) had relatively minor effects. PDGF, EGF or FGF increased cardiomyocyte size via ERK1/2, whereas insulin, IGF-1 or NGF had no effect suggesting minimum thresholds/durations of ERK1/2 signaling are required for the morphological changes associated with hypertrophy. Peptide growth factors are widely accepted to activate phospholipase C γ1 (PLCγ1) and protein kinase C (PKC). In cardiomyocytes, only PDGF stimulated tyrosine phosphorylation of PLCγ1 and nPKCδ. Furthermore, activation of ERK1/2 by PDGF, but not EGF, required PKC activity. In contrast, EGF substantially increased Ras.GTP with rapid activation of c-Raf, whereas stimulation of Ras.GTP loading by PDGF was minimal and activation of c-Raf was delayed. Our data provide clear evidence for differential coupling of PDGF and EGF receptors to the ERK1/2 cascade, and indicate that a minimum threshold/duration of ERK1/2 signaling is required for the development of cardiomyocyte hypertrophy.


*Keywords:* Peptide growth factors; Phospholipase C; ERK cascade

## 1. Introduction

Ventricular cardiomyocytes withdraw from the cell cycle in the perinatal period becoming terminally differentiated. In the adult heart, ventricular cardiomyocytes respond to an increase in workload with a hypertrophic growth response associated with changes in morphology (increases in cell size and myofibrillar content) and gene expression [including re-expression of genes which are normally expressed only early in development (e.g. atrial natriuretic factor)] [1]. The mechanisms associated with hypertrophy have been investigated over many years. It is probable that locally released neurohumoral factors stimulate the cardiomyocytes, initiating intracellular signaling pathways which elicit cardiomyocyte hypertrophy [2]. Principal factors appear to be those which primarily stimulate Gq protein-coupled receptors (GqPCRs) and include endothelin-1 (ET-1) and α-adrenergic agonists. Consequently, much research has focused on the intracellular signaling pathways activated by GqPCR agonists in cardiomyocytes.

As in other systems, ET-1 stimulates phospholipase Cβ (PLCβ) activity in cardiomyocytes resulting in the hydrolysis of phosphatidylinositol 4,5 *bis*phosphate to produce inositol 1,4,5 *tris*phosphate [3] and diacylglycerol (DAG) [4]. There is consequent activation of DAG-responsive novel protein kinase C (PKC) isoforms, nPKCδ and nPKCε, as measured by their translocation from the soluble to the particulate fraction of the cell [5]. These and other data implicate PKC activity in cardiomyocyte hypertrophy [2]. GqPCR agonists also activate mitogen-activated protein kinases (MAPKs) in

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doi:10.1016/j.cellsig.2005.04.005

cardiomyocytes in a PKC-dependent manner [2]. The three best-characterised MAPKs [extracellular signal-regulated kinases 1/2 (ERK1/2), the c-Jun N-terminal kinases and p38-MAPKs] are all implicated in cardiomyocyte hypertrophy, although ERK1/2 are particularly strongly implicated in the response [6]. Although most studies of intracellular signaling in cardiomyocyte hypertrophy have examined the response to GqPCR agonists, cardiomyocytes also respond to peptide growth factors which activate receptor protein tyrosine kinases (RPTKs). Thus, acidic fibroblast growth factor (aFGF) activates ERK1/2 in neonatal rat cardiomyocytes, the upstream MAPK kinases (MKK1/2) and the MAPK kinase kinase c-Raf [7,8]. Other peptide growth factors including epidermal growth factor (EGF [9]), platelet-derived growth factor (PDGF [10]), and insulin-like growth factor 1 (IGF-1 [11]) also activate ERK1/2 in cardiomyocytes, and it is generally accepted that such agonists promote cardiomyocyte hypertrophy.

The mechanisms by which RPTKs activate ERK1/2 in proliferating cells have been investigated extensively [12]. In essence, growth factors activate their RPTK leading to phosphorylation of the receptor and/or a docking protein. Grb2 is recruited to the receptor complex and is associated with Sos (a guanine nucleotide exchange factor for the small G protein Ras) which is brought into the vicinity of Ras. Ras becomes activated and recruits c-Raf to the membrane for activation, initiating the ERK1/2 cascade. Tyrosine phosphorylation of RPTKs and/or adapter molecules also leads to the activation of other pathways including phosphoinositide 3-kinase (PI3K) and PLCγ. PI3K activity leads to activation of protein kinase B (PKB, also known as Akt), whereas PLCγ hydrolyses phosphatidylinositol 4,5 *bis*phosphate presumably leading to activation of DAG-responsive PKC isoforms. The activation of ERK1/2, PKB/Akt and PLCγ by IGF-1, PDGF, EGF and FGF have all been reported in a variety of different cell types [11,13–16], but the overall response of cardiomyocytes has not been examined. Since, in other cells, the duration and intensity of activation of, for example, the ERK1/2 cascade, can influence such global responses as proliferation vs. differentiation [17], the precise wiring of the pathways and relative activation of different components is likely to have a significant impact on cardiomyocyte function. Here, we demonstrate that the degree of ERK1/2 activation is an important factor in the development of cardiomyocyte hypertrophy, and illustrate that PDGF signaling to the ERK1/2 cascade differs substantially from that of other growth factors.

## 2. Materials and methods

### 2.1. Cardiomyocyte cultures

Ventricular myocytes, prepared from 1–2 day Sprague–Dawley rats, were plated at $4 \times 10^6$ cells/60 mm dish (18 h) [18]. Serum was withdrawn (24 h). To examine the role of PKC, myocytes were pretreated with PMA (1 μM, 24 h) or GF109203X (10 μM, 15 min). Agonists (PDGF AB, 20 ng/ml; aFGF, 20 ng/ml; IGF-1, 75 ng/ml; insulin, 50 mU/ml; NGF, 50 ng/ml; ET-1, 100 nM; PMA, 1 μM) were added to the medium. Peptide growth factors and PMA were from Sigma Aldrich Chemical Co. Human Actrapid insulin was from Nova Nordisk Pharmaceuticals Limited. GF109203X was from Alexis.

### 2.2. Activation of signaling pathways

Total myocyte extracts were prepared, and phosphorylated/total ERK1/2 or PKB were analysed by immunoblotting as described [19], using phosphospecific or total ERK1/2 or PKB polyclonal antibodies (Cell Signaling, 1/1000). ERK1/2 activity was measured by in-gel protein kinase assays with myelin basic protein (Upstate) as substrate [5]. Hydrolysis of phosphatidylinositols was measured as described [3].

To assess tyrosine phosphorylation of PLCγ1 or PKCδ, cells ($4 \times 10^6$) were scraped into Buffer A [20 mM Tris–HCl (pH 7.4), 2 mM EDTA, 100 mM KCl, 5 mM NaF, 0.2 mM $Na_3VO_4$, 2 μM microcystin, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 0.5% (v/v) 2-mercaptoethanol, 10 mM benzamidine, 0.2 mM leupeptin, 0.01 mM *trans*-epoxy succinyl-L-leucylamido-(4-guanidino)butane, 0.3 mM phenylmethylsulphonyl fluoride] and centrifuged (10,000×*g*, 5 min). The supernatants were incubated (1 h, 4 °C) with monoclonal anti-phosphotyrosine antibodies (PY99; 2 μg) prebound to Protein G-Sepharose. Immunoprecipitates were washed, resuspended in 15 μl buffer A and boiled with 0.33 M Tris–HCl pH 6.8, 10% (w/v) SDS, 13% (v/v) glycerol, 133 mM dithiothreitol (15 μl). Samples were immunoblotted using 8% (w/v) polyacrylamide gels and polyclonal primary antibodies to PKCδ (1/400) or PLCγ1 (1/200). Antibodies were from Santa Cruz Biotechnology Inc.

The activity of immunoprecipitated c-Raf was assayed as described [20] using 0.2 μg GST-MKK1 as substrate. Samples were immunoblotted with antibodies to phosphorylated MKK1/2 (Cell Signaling, 1/1000) or total c-Raf (1/1000). Ras.GTP was affinity purified as described [21] and immunoblotted with monoclonal antibodies for total Ras (1/500), K-Ras (1/200) or N-Ras (1/100), or polyclonal antibodies for H-Ras (1/200). Antibodies to total Ras (R02120) or c-Raf (R19120) were from BD Biosciences. Antibodies to H-Ras (C20), K-Ras (F234) or N-Ras (F155) were from Santa Cruz Biotechnology Inc.

For all immunoblotting, monoclonal antibodies were amplified with rabbit anti-mouse immunoglobulin secondary antibodies, and detected with HRP-conjugated tertiary antibodies, whereas polyclonal antibodies were detected with HRP-conjugated secondary antibodies. Secondary/tertiary antibodies were from Dako. Bands were detected by enhanced chemiluminescence (Santa

Cruz Biotechnology Inc.). Scanning densitometry (Image-master 1D calibrated with Kodak photographic step tablet no. 2; Amersham Biosciences) was used for semi-quantitative analysis of immunoblot data and in-gel kinase assays.

### 2.3. Immunostaining and planimetry

Myocytes were plated ($10^5$ cells/well, 18 h) in Permanox four-well chamber slides precoated with gelatin and laminin [22]. Serum was withdrawn (24 h) and myocytes were

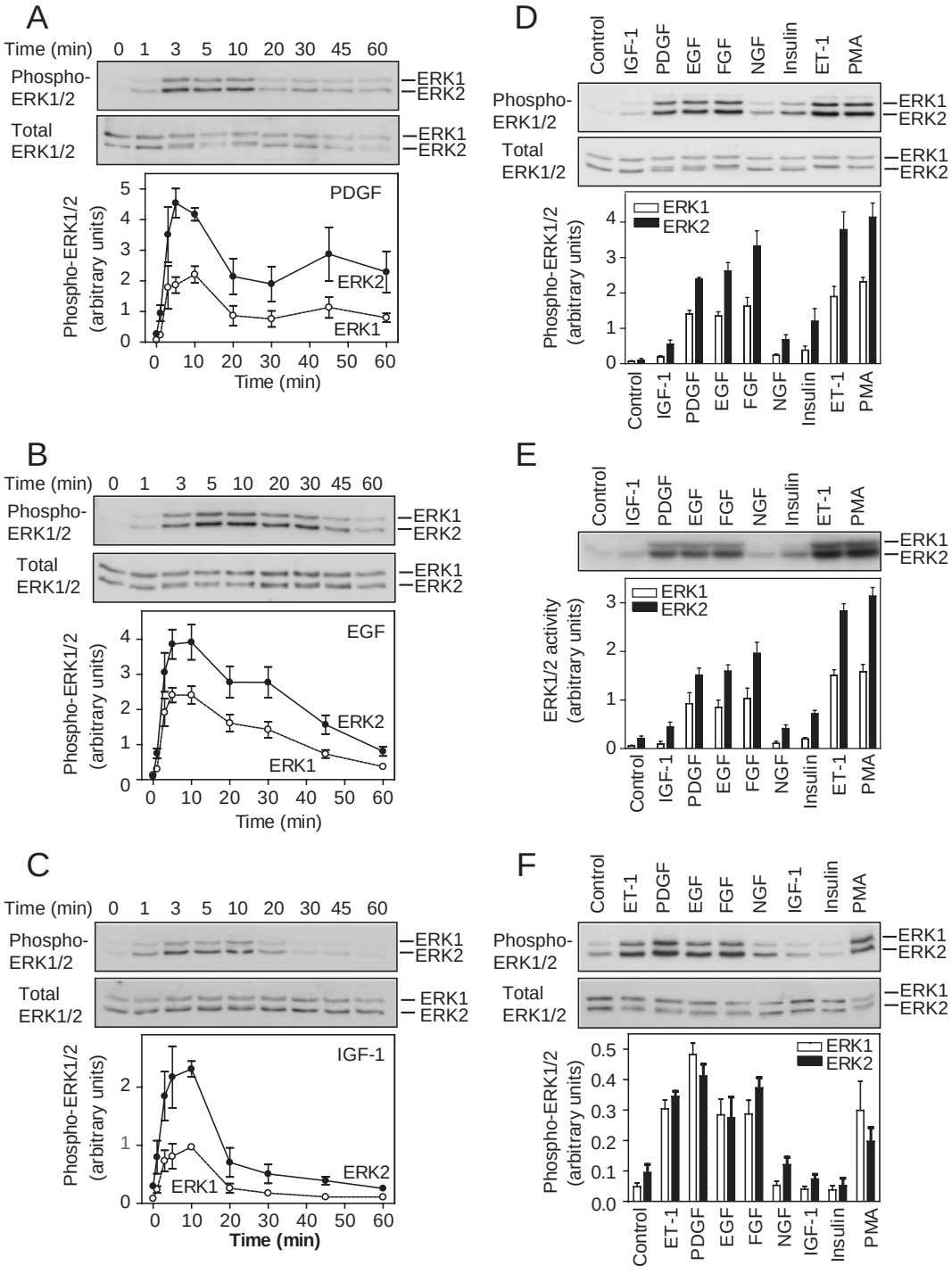


Fig. 1. Activation of ERK1/2 by peptide growth factors in cardiomyocytes. Myocytes were unstimulated (Control), exposed to PDGF (A), EGF (B) or IGF-1 (C) for the times indicated, or exposed to the indicated agonists for 5 (D, E) or 60 (F) min. (A–D and F) Myocyte extracts were immunoblotted for phosphorylated (upper images) or total (lower images) ERK1/2. (E) ERK1/2 activity in myocyte extracts was measured by in-gel kinase assay with myelin basic protein as substrate. Bands were analysed by scanning densitometry (lower panels). Results are means±S.E. for 3 (A–C, F) or 4 (D, E) independent experiments.

exposed (24 h) to agonists in the absence or presence of U0126 (10 μM). Following immunostaining with antibodies to β-myosin heavy chain (Vector Laboratories) as described [22], digital planimetry was used to measure cardiomyocyte area (Scion Image Beta 4.02, Scion Corporation USA). >150 myocytes were measured for each condition in each experiment. Results were normalised to the mean area of control cells for each experiment. Statistical analysis (one-way ANOVA with Tukey's multiple comparison test) was with GraphPad Prism.

## 3. Results

### *3.1. Activation of ERK1/2 and PKB/Akt by peptide growth factors in cardiomyocytes*

We investigated the activation of ERK1/2 by growth factors [PDGF AB, EGF or IGF-1; maximally effective concentrations (data not shown)] in cardiomyocytes for comparison with the response to ET-1 or PMA (a phorbol ester which activates DAG-responsive PKC isoforms directly). PDGF, EGF or IGF-1 promoted a rapid (maximal within 3–5 min) increase in phosphorylation (activation) of ERK1/2 as assessed by immunoblotting with phosphospecific antibodies (Fig. 1A–C). However, the subsequent kinetics differed. The increase in phospho-ERK1/2 in response to PDGF declined substantially at 10–20 min, although levels remained elevated for at least 60 min (Fig. 1A). In response to EGF, there was a gradual decline in phospho-ERK1/2 from ~10 to 60 min, although levels remained above basal even at 60 min (Fig. 1B). In contrast, the response to IGF-1 was clearly transient and levels of phospho-ERK1/2 returned to basal within 30 min (Fig. 1C).

The degrees of stimulation of ERK1/2 by PDGF, EGF, IGF-1 and NGF were compared directly with those induced by aFGF, insulin, ET-1 and PMA by immunoblotting with phosphospecific antibodies (Fig. 1D and F) or using in-gel protein kinase assays with myelin basic protein as substrate to measure activity directly (Fig. 1E). The response was

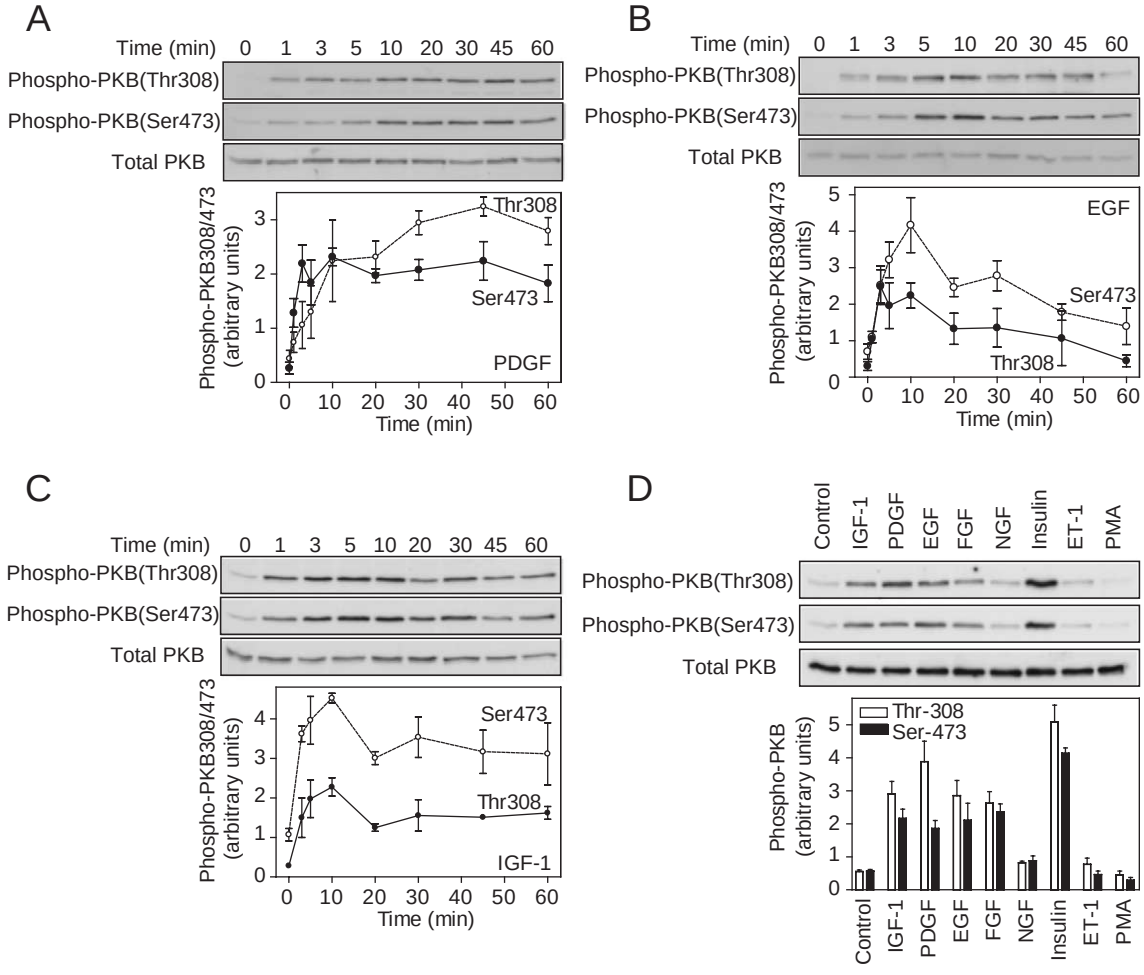


Fig. 2. Activation of PKB/Akt by peptide growth factors in cardiomyocytes. Myocytes were unstimulated (Control), exposed to PDGF (A), EGF (B) or IGF-1 (C) for the times indicated, or exposed to the indicated agonists for 5 min (D). Protein extracts were immunoblotted for PKB/Akt phosphorylated on Thr308 (upper images) or Ser473 (middle images), or for total PKB/Akt (lower images). Bands were analysed by scanning densitometry (lower panels). Results are means ± S.E. for 3 (A–C) or 4 (D) independent experiments.

examined at 5 min (the time at which maximal activation was seen by all agonists) in order to compare the degree of activation, and at 60 min to gauge the sustainability of the response. At 5 min, using in-gel kinase assays (Fig. 1E), the degree of ERK1/2 activation was such that ET-1 or PMA promoted an approximately similar response (29- to 35-fold for ERK1; 37- to 40-fold for ERK2) which was greater than that induced by PDGF, EGF or FGF (21- to 25-fold for ERK1; 24- to 32-fold for ERK2), each of which induced a similar degree of ERK1/2 activation. The response to PDGF, EGF or FGF was greater than that induced by insulin (~6-fold for ERK1; ~12-fold for ERK2) and the weakest activation of ERK1/2 was observed with IGF-1 or NGF (~3-fold for ERK1; ~6-fold for ERK2). This was supported by calibrated densitometry of the phospho-ERK1/2 immunoblots (Fig. 1D). Phosphorylated ERK2 has reduced mobility with SDS-PAGE and the active form is observed as a band of higher relative molecular mass on total ERK2 immunoblots. The proportion of this form of ERK2 correlated directly with the phospho-ERK1/2 blots and in-gel kinase assays (Fig. 1D and E). At 60 min, the increase in ERK1/2 phosphorylation had declined substantially for all agonists (Fig. 1F). However, the degree of phosphorylation of ERK1/2 remained above basal levels for PMA, ET-1, PDGF, EGF or FGF (6- to 10-fold), but not insulin, IGF-1 or NGF (Fig. 1F). The activation of ERK1/2 by insulin, IGF-1 or NGF is therefore not only to a lesser degree than that induced by the other agonists, but the response is also not as sustained.

Since growth factors, particularly insulin and IGF-1, stimulate PI3K and activate PKB/Akt, we examined and compared the phosphorylation of PKB/Akt by the various stimuli by immunoblotting with phosphospecific antibodies to each of the two sites required for activity (Thr308 and Ser473 for mouse PKBα). PDGF, EGF or IGF-1 promoted a rapid (1–3 min) increase in phosphorylation of PKB/Akt on Thr308 and Ser473 (Fig. 2A–C). NGF did not induce any significant increase in phosphorylation of PKB/Akt (results not shown). The response to PDGF was sustained over at least 60 min (Fig. 2A), whereas the response to EGF was maximal at 5–10 min and then declined (Fig. 2B). The response to IGF-1 was also maximal at 5–10 min and, although there was subsequently some decline, levels of phosphorylated PKB/Akt remained elevated for at least 60 min (Fig. 2C). Direct comparison of the effects of different growth factors, ET-1 or PMA on the phosphorylation of PKB/Akt at 5 min (the time at which maximal activation was seen by all agonists which signal through PKB/Akt) revealed that insulin had the greatest effect on PKB/Akt phosphorylation (Fig. 2D). The response to insulin was greater than that induced by PDGF, EGF or FGF which all promoted a similar degree of phosphorylation of PKB/Akt and this was greater than the response to NGF (Fig. 2D). Consistent with previous studies [23], there was minimal effect of ET-1 on the phosphorylation of PKB/Akt and PMA had no measurable effect. These experiments confirm the efficacy of insulin and IGF-1 despite the minimal activation of ERK1/2 (Fig. 1).

### 3.2. Differential activation of phospholipase C and PKCδ by RPTKs

PDGF, EGF and IGF-1 receptors all have the potential to activate PLCγ leading to hydrolysis of phosphatidylinositol 4,5 *bis*phosphate to produce DAG and inositol 1,4,5 *tris*phosphate [11,14,16]. We examined the stimulation of PLC by these growth factors in cardiomyocytes pre-labelled with [$^3$H]inositol. Production of total [$^3$H]inositol phosphates (InsPs, as a measure of PLC activity) was measured. PDGF promoted a gradual increase in [$^3$H]InsPs from 1 to 3 min which was approximately linear over at least 15 min (Fig. 3A). Surprisingly, of the growth factors studied, only PDGF promoted phosphoinositide hydrolysis (Fig. 3B). However, the response to PDGF was less than that induced by ET-1 which, acting through its GqPCR, stimulates PLCβ isoforms [24]. There was no change in production of [$^3$H]InsPs in unstimulated cells during the assay period (data not shown).

PLCγ isoforms (PLCγ1 and PLCγ2) are activated by tyrosine phosphorylation [24]. To examine this in cardiomyocytes, phosphotyrosine-containing proteins were immunoprecipitated and then immunoblotted for PLCγ1/2. PDGF potently and rapidly (from ~1 min) promoted tyrosine phosphorylation of PLCγ1 (Fig. 4A). EGF and, to an even

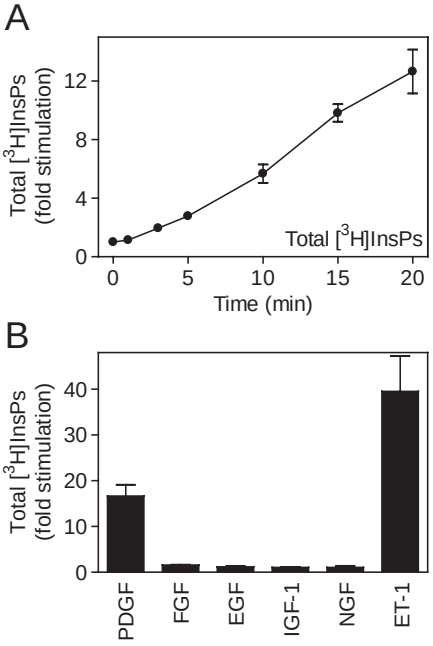


Fig. 3. PDGF, but not other peptide growth factors, stimulates hydrolysis of phosphatidylinositols. Myocytes were pre-labelled with [$^3$H]inositol (24 h) then unstimulated, exposed to PDGF for the times indicated (A) or exposed to the indicated agonists for 20 min (B), in the presence of 10 mM LiCl. [$^3$H]InsPs were isolated by anion exchange chromatography and measured by liquid scintillation counting. Results are means ± S.E. for at least 3 independent experiments.

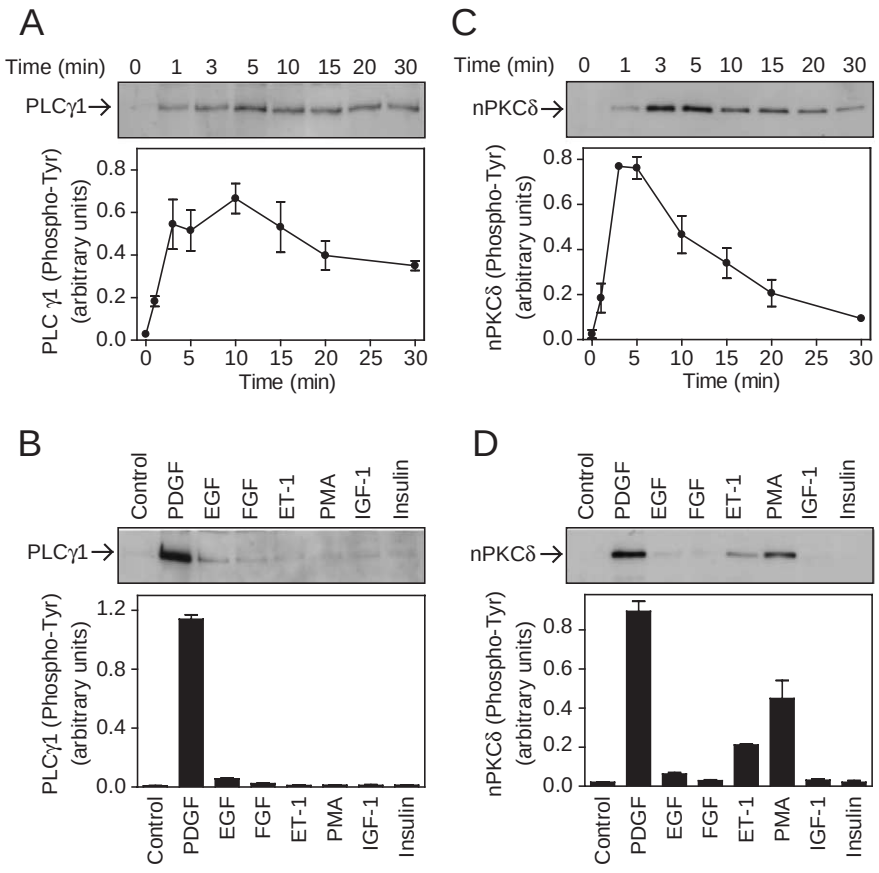


Fig. 4. PDGF promotes tyrosine phosphorylation of PLCγ1 and nPKCδ. Myocytes were unstimulated (Control), exposed to PDGF for the times indicated (A, C) or to the indicated agonists for 5 min (B, D). Phosphotyrosine-containing proteins were immunoprecipitated from the extracts and were immunoblotted with antibodies to PLC1γ (A, B) or nPKCδ (C, D). Representative images are shown in the upper panels. Bands were analysed by scanning densitometry (lower panels). Results are means±S.E. for 3 independent experiments (A, C) or means±S.D. for 2 independent experiments (B, D).

lesser extent, aFGF marginally increased tyrosine phosphorylation of PLCγ1, but IGF-1, insulin, ET-1 or PMA had no effect (Fig. 4B). No tyrosine phosphorylated PLCγ2 was detected in phosphotyrosine immunoprecipitates from cells exposed to any agonist studied, although PLCγ2 was identified in total cardiomyocyte extracts (data not shown).

Of the two products of PLC activity, DAG is the established physiological activator of classical (α, β, and γ) and novel (δ, ε, η, θ) PKCs [25]. By binding to DAG, these PKCs translocate from the soluble to the particulate fraction of the cell. This translocation is commonly used to assess c/nPKC activation. cPKCα, nPKCδ and nPKCϵ all translocate to the particulate fraction of cardiomyocytes in response to PMA (which mimics DAG) [2]. Although PLCγ was activated in cardiomyocytes exposed to PDGF (Fig. 4A) with an associated increase in phosphoinositide hydrolysis (Fig. 3), translocation of cPKCα, nPKCδ or nPKCε was not detected in cardiomyocytes following stimulation with PDGF (data not shown). However, PKCs are also regulated by reversible phosphorylation and nPKCδ, in particular, is regulated by tyrosine phosphorylation [26]. To examine this, immunoprecipitated phosphotyrosine-containing proteins from cardiomyocytes were immunoblotted for nPKCδ. PDGF promoted a rapid (within 1–3 min) and substantial increase in tyrosine phosphorylation of nPKCδ which was maximal at 3–5 min and declined to basal levels after ~30 min (Fig. 4C). The response to PDGF was greater than that induced by PMA (previously reported to increase tyrosine phosphorylation of nPKCδ [26]) or ET-1 (Fig. 4D). EGF, FGF, IGF-1 and insulin had a minimal effect on tyrosine phosphorylation of nPKCδ in cardiomyocytes (Fig. 4D), consistent with the negligible effects of these agonists on tyrosine phosphorylation of PLCγ1 (Fig. 4B) and on phosphoinositide hydrolysis (Fig. 3B). Thus, in an endogenous setting in primary cardiomyocytes, signaling through PLCγ and nPKCδ by RPTKs is segregated.

### 3.3. Signaling to ERK1/2 by PDGF vs. EGF

In cardiomyocytes, activation of ERK1/2 by ET-1 is PKC-dependent and is inhibited by selective pharmacological inhibitors or by prolonged treatment with PMA to downregulate c/nPKCs [2]. As expected, given that there was no indication of activation of PLCγ or nPKCδ (Fig. 4), the activation of ERK1/2 [assessed by immunoblotting with phosphospecific antibodies (Fig. 5A) or in-gel protein kinase assays (Fig. 5B)] by EGF was not inhibited by the

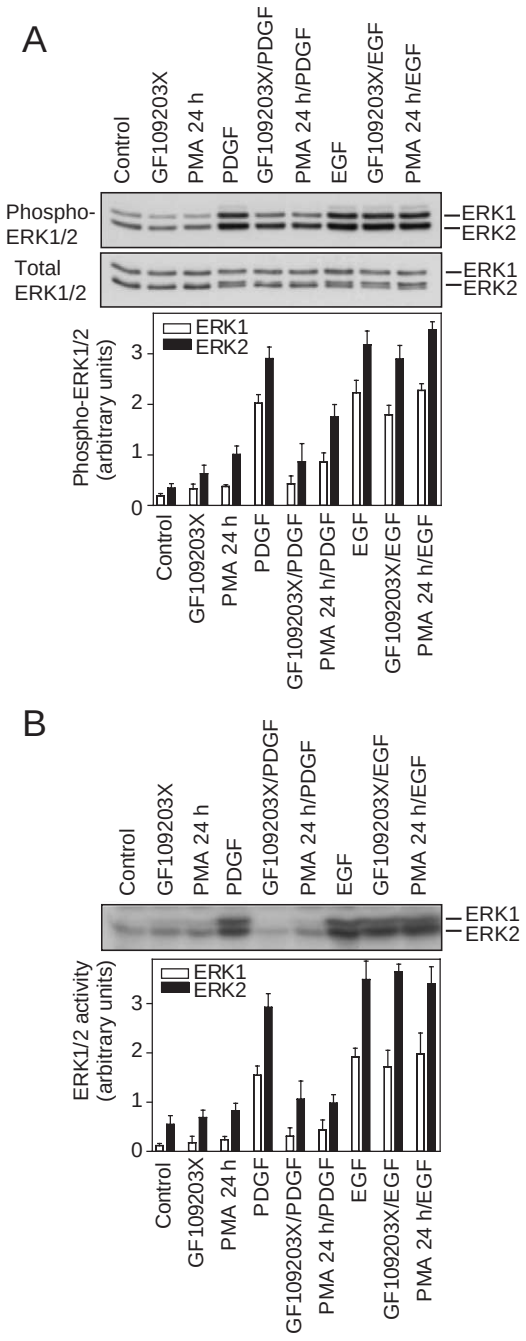


Fig. 5. Activation of ERK1/2 by PDGF, but not EGF, requires PKC activity. Myocytes were unstimulated (Control) or exposed to PDGF or EGF in the absence or presence of GF109203X or PMA (24 h pretreatment). (A) Myocyte extracts were immunoblotted for phosphorylated (upper image) or total (lower image) ERK1/2. (B) ERK1/2 activity in myocyte extracts was measured by in-gel kinase assay with myelin basic protein as substrate. Bands were analysed by scanning densitometry (lower panels). Results are means ± S.E. for 4 independent experiments.

PKC inhibitor, GF109203X (10 μM) or by pretreatment (24 h) with 1 μM PMA to downregulate c/nPKCs (Fig. 5). However, either treatment suppressed the activation of ERK1/2 by PDGF (Fig. 5). As indicated above, the active form of ERK2 is observed as a band of higher relative molecular mass on total ERK2 immunoblots. The proportion of this form of ERK2 correlated directly with the phospho-ERK1/2 blots and the in-gel kinase assays. It is therefore clear that, although PDGF and EGF induce similar activation of ERK1/2, the wiring of the pathway is different.

The ERK1/2 cascade is a three-tiered cascade in which Raf phosphorylates and activates MKK1/2 which, in turn, phosphorylate and activate ERK1/2 [2]. We compared the activation of c-Raf by PDGF and EGF. As expected, since both agonists are equipotent in activating ERK1/2 (Fig. 1), PDGF or EGF activated c-Raf to a similar degree (maximal at ~3 min) (Fig. 6A and B). However, the activation of c-Raf by EGF was apparent within 1 min of stimulation when there was no detectable activation by PDGF at this time. This suggested that the coupling from Ras to c-Raf may be different. The activation (GTP-loading) of total Ras isoforms (Fig. 6C) and specific activation of H-Ras (Fig. 6D), K-Ras (Fig. 6E) or N-Ras (Fig. 6F) was examined. Surprisingly, given that there has been no previous indication of differential coupling of RPTKs to Ras and the ERK1/2 cascade, the stimulation of total Ras.GTP or of GTP-loading of the individual Ras isoforms by PDGF was minimal and substantially less than that induced by EGF. Thus, in cardiomyocytes in which endogenous receptors are signaling through endogenous intermediates, coupling of PDGF to the ERK1/2 cascade differs markedly from that of EGF, with less activation of Ras isoforms but a requirement for PKC activity.

### 3.4. Effects of growth factors on cardiomyocyte morphology

The ERK1/2 cascade is required for cardiomyocyte hypertrophy induced by GqPCR agonists although activation of ERK1/2 alone may not be sufficient for the full response [2]. We determined whether activation of ERK1/2 by different peptide growth factors [given that the degree and time course of ERK1/2 activation differs between them (Fig. 1)] is sufficient for the morphological changes associated with myocyte hypertrophy. Cardiomyocytes were exposed to growth factors or ET-1 for 24 h in the absence or presence of 10 μM U0126 (which inhibits MKK1/2) and the morphology examined following immunostaining for β-myosin heavy chain (Fig. 7). Consistent with previous studies [19,27], ET-1 promoted changes in cardiomyocyte morphology associated with hypertrophy (increases in cell size, increased cell–cell contacts and increased immunostaining for myofibrillar proteins) and the response was attenuated by U0126 (Fig. 7B and F). EGF (Fig. 7C), PDGF (Fig. 7D) or aFGF (Fig. 7J), which substantially activated the ERK1/2 cascade (Fig. 1), also promoted the morphological changes associated with cardiomyocyte hypertrophy with a significant increase in cell area (Fig. 7M). U0126 abolished the morphological changes induced by EGF or PDGF (Fig. 7G, H and N). Although insulin, IGF-1 or NGF activated ERK1/2 to a degree, this appeared insufficient to promote the morphological changes associated with cardiomyocyte hypertrophy (Fig. 7K, L and M). Our data are

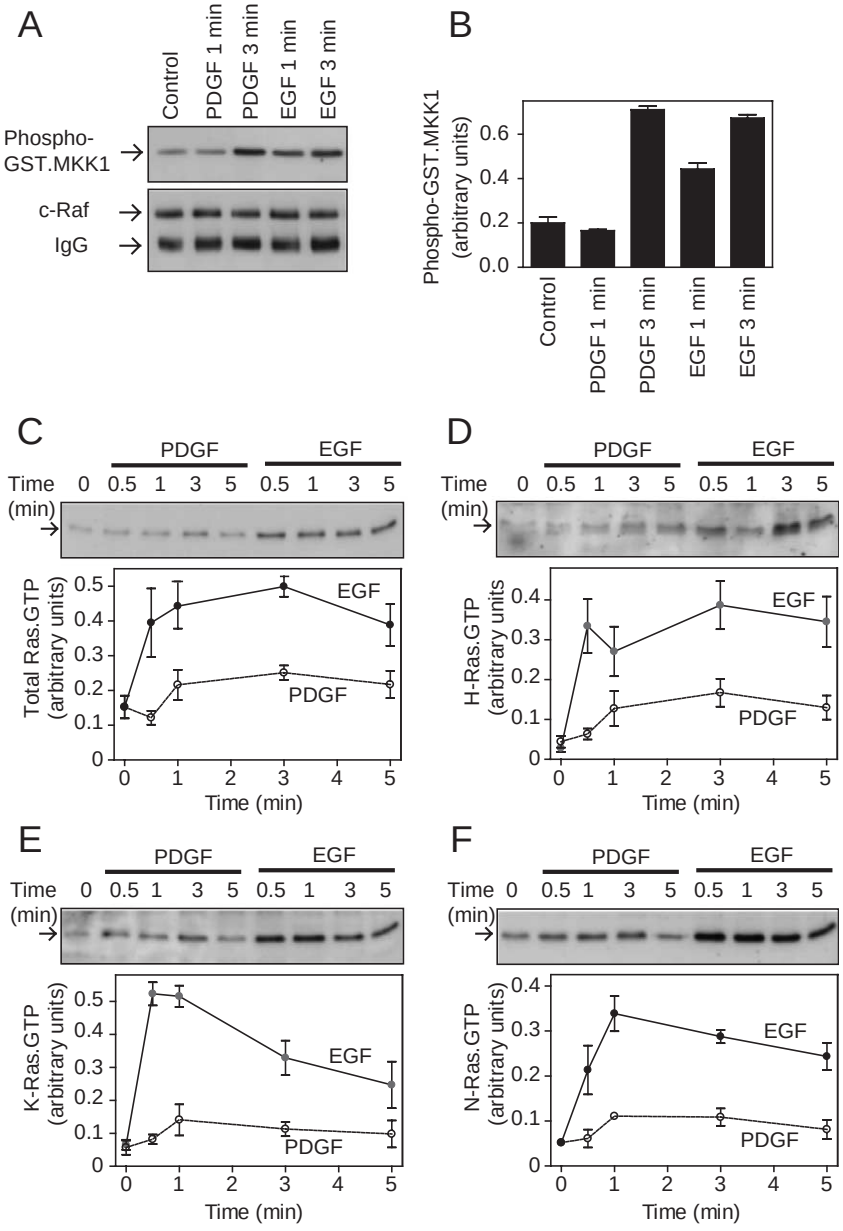


Fig. 6. EGF, but not PDGF, potently activates Ras and promotes early activation of c-Raf. Myocytes were unstimulated or exposed to PDGF or EGF for the times indicated. (A and B) c-Raf was immunoprecipitated from myocyte extracts and c-Raf activity was assayed in vitro using GST-MKK1 as substrate. The assay mix was immunoblotted with antibodies to phosphorylated MKK1 (A, upper image) or c-Raf (A, lower image). Bands were analysed by scanning densitometry (B). Results are means ± S.E. for 3 independent experiments. (C–F) Ras.GTP was purified from myocyte extracts using the Ras binding domain of c-Raf, and immunoblotted with antibodies to total Ras (C), H-Ras (D), K-Ras (E) or N-Ras (F). Representative images are shown in the upper panels. Bands were analysed by scanning densitometry (lower panels). Results are means ± S.E. for 3 (E and F) or 4 (C and D) independent experiments.

consistent with the concept that there is a minimum threshold and/or duration of ERK1/2 signaling required to promote cardiomyocyte hypertrophy.

## 4. Discussion

Over many years, many studies have examined the intracellular signaling pathways which are activated by peptide growth factor receptors. The approaches are often based on an appropriate cell line (e.g. one which readily responds to the stimulus and can be readily grown in tissue culture) and, more recently, usually involve interventionist procedures such as overexpression of receptors or signaling intermediates which may be mutated into constitutively activated or dominant-negative forms. Such approaches are highly relevant for disease states such as cancer in which this scenario occurs. However, any overexpression system undoubtedly disrupts the stoichiometry of signaling components within the cell, potentially leading to inappropriate interactions and crosstalk. Furthermore, the wiring of intracellular signaling in a proliferating cell in culture may

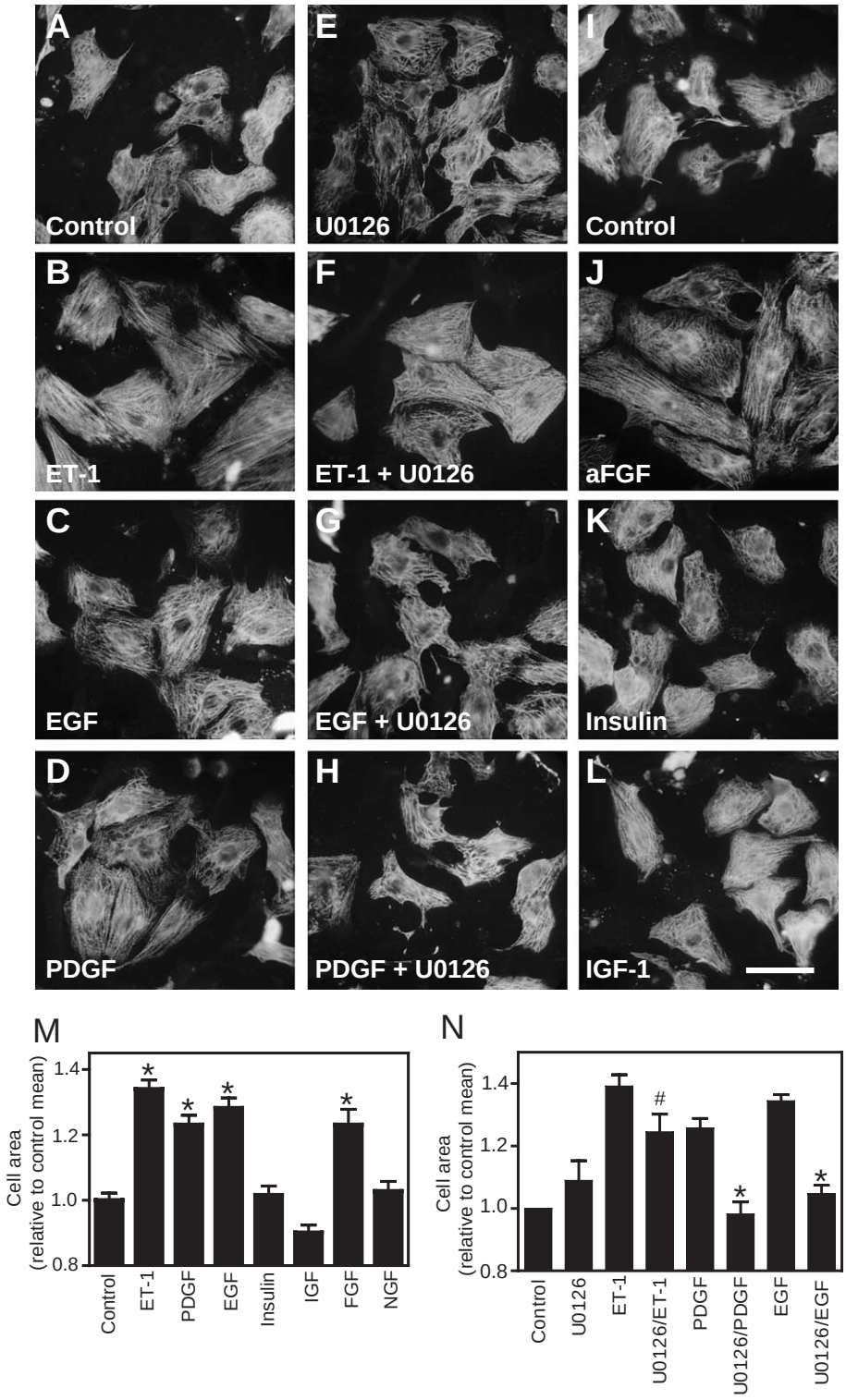


Fig. 7. Activation of ERK1/2 is associated with the morphological changes associated with cardiomyocyte hypertrophy. Myocytes were unstimulated (Control) or exposed to the indicated agonists in the absence or presence of U0126 for 24 h. Cells were immunostained for β-myosin heavy chain. (A–L) Representative images of cardiomyocytes. Bar = 10 μm. (M and N) Myocyte cell area was measured using Scion Image software. (M) A representative experiment to show the effects of different agonists of myocyte cell area. Results are means ± S.E. ($n > 150$); *$p < 0.001$ (relative to unstimulated cells). The experiment was repeated twice more with similar results. (N) Effects of U0126 on the increase in cell size induced by ET-1, PDGF or EGF. Results are means ± S.E. for 3 independent experiments (each with $n > 150$), taking the mean size for each experiment as a single observation. #$p < 0.05$ (relative to cells stimulated with ET-1); *$p < 0.001$ relative to cells stimulated with PDGF or EGF.

not necessarily be representative of "normal" cells in the body which are not necessarily continually dividing. For a highly specialised cell such as a terminally differentiated cardiomyocyte, it is likely that the signaling is very specific. Our data clearly indicate that this is the case. Thus, although PDGF, EGF and aFGF all activated the ERK1/2 cascade to a similar degree (Fig. 1D and E), only PDGF significantly stimulated tyrosine phosphorylation of PLCγ1 (Fig. 4B) with an associated increase in phosphoinositide-PLC activity (Fig. 3B) and tyrosine phosphorylation of nPKCδ (Fig. 4D). Furthermore, the activation of ERK1/2 by PDGF, but not EGF or FGF, required PKC activity (Fig. 5; [7]) and did not correlate with Ras activation (Fig. 6).

In other cells, PDGF, EGF or FGF can all activate PLCγ1 [13,15,16] potentially leading to production of DAG and activation of DAG-responsive PKCs. The reason for specific signaling from PDGF (rather than EGF or FGF) receptors to PLCγ1 in cardiomyocytes is not immediately obvious, but compartmentalisation of signaling components (which may be specific for the cardiomyocyte) is likely to be a factor. It should be noted that the cardiomyocytes used in this study were primary, terminally differentiated cells which were largely unmanipulated, so it is likely that the signaling is more representative of the situation in vivo than cells in which signaling elements are altered. Although, in non-myocytes, there seems limited evidence that IGF-1 or insulin stimulate PLCγ, it has been reported that PLCγ1 is tyrosine phosphorylated in cardiomyocytes in response to IGF-1 [11] and that IGF-1 stimulates the production of $InsP_3$ [28]. Our data are entirely inconsistent with these studies since we never observe any significant increase in tyrosine phosphorylation of PLCγ1 or phosphoinositide hydrolysis in cardiomyocytes exposed to IGF-1 (Fig. 3B). This is difficult to explain given that the same cell culture system was used. An obvious difference is the source of IGF-1, but both studies used recombinant peptides.

Stimulation of cardiomyocytes with GqPCR agonists such as ET-1 or bradykinin is associated with PLC activity and activation of nPKCδ or nPKCε (as measured by their transient translocation to the particulate fraction of the cell) [5,22]. Although PDGF stimulated PLC activity in cardiomyocytes (Fig. 3), we consistently failed to detect any translocation of PKC isoforms to the particulate fraction of the cell (data not shown). However, we did observe an increase in tyrosine phosphorylation of nPKCδ (Fig. 4C) suggesting that there was activation of this (and possibly other) DAG-responsive PKC isoform in response to PDGF. The apparent lack of translocation of PKCs to the membrane may reflect the difference in time course of activation. Thus, PDGF promoted a much slower (detected at 1–3 min) and more gradual accumulation of InsPs (Fig. 3A) and $InsP_3$ (data not shown) compared with ET-1 or bradykinin which induce a very rapid peak of $InsP_3$ release (maximal at ~15 s) [3]. Assuming that, as with ET-1 or bradykinin, any translocation is transient, the gradual release of DAG in response to PDGF may be insufficient for global translocation of PKC isoforms to be detected.

One aspect of the PDGF response in cardiomyocytes of particular interest is the delay in activation of c-Raf relative to activation by EGF (Fig. 6A and B) and the relatively poor activation of Ras isoforms (Fig. 6C–F). Although the small degree of Ras.GTP-loading induced by PDGF may be sufficient for c-Raf activation, given that EGF substantially activates c-Raf at 1 min with no activation by PDGF, whereas the degree of activation by the two agonists at 3 min is similar, it is possible that a second input links PKC directly to c-Raf. In any case, signaling from the receptor to c-Raf clearly differs for PDGF and EGF, with the PDGF receptor apparently requiring a signal from PKC to Ras and/or c-Raf rather than a direct signal via the established route of Grb2/Sos. The reduced degree of Ras activation by PDGF presumably has consequences for signaling via other Ras-interacting proteins such as Ral.GDS or PI3K, although this remains to be investigated.

The data in this study address issues relating to the wiring of intracellular signaling pathways, but also have considerable bearing on the role(s) of the pathways in hypertrophic growth of cardiomyocytes. Our data are consistent with previous studies implicating the ERK1/2 cascade in the hypertrophic response [2], since (as has been shown previously for GqPCR signaling to hypertrophy [27]) inhibition of this pathway suppresses the morphological changes associated with cardiomyocyte hypertrophy (Fig. 7). However, whereas ET-1, FGF, PDGF or EGF promote substantial activation of ERK1/2 and induce an increase in cardiomyocyte size, IGF-1 or insulin are less potent at activating this pathway and did not cause the morphological changes associated with hypertrophy (Figs. 1 and 7). This suggests that, although ERK1/2 can promote cardiomyocyte growth, the degree and/or duration of activation may dictate the overall response. Alternatively, an additional input from other pathways may be required.

As expected, IGF-1 and insulin (and the other growth factors) potently activated PKB/Akt confirming that the pathway is operative in cardiomyocytes (Fig. 2). Other studies have suggested that IGF-1 induces hypertrophic growth in cardiomyocytes [29], potentially through PKB/Akt signaling to inhibit GSK3 activity [30]. Consistent with this, IGF-1 or insulin both promote changes in protein turnover, overexpression of PKB/Akt in transgenic mice results in enlarged hearts, and inhibition of the pathway suppresses cardiomyocyte growth [31,32]. However, the interpretation of the results probably depends on the question posed in the study. The PKB/Akt pathway is clearly cytoprotective and regulates protein synthesis in all systems studied including cardiomyocytes [32]. It is therefore perhaps not surprising that inhibiting the pathway suppresses cardiac hypertrophy, or that long-term overexpression of PKB/Akt in the context of a transgenic mouse causes an increase in protein content and cell size. Whether

or not IGF-1 or insulin promotes the full hypertrophic response is still open to debate. Our data indicate that, at least in the short term (24 h), acute activation of PKB/Akt by IGF-1 or insulin is in itself insufficient to induce the morphological changes associated with cardiomyocyte hypertrophy. It is possible that the hypertrophic changes would become apparent over a longer period of time, which could account for the apparent discrepancy between this and other studies. However, serum starvation of cardiomyocytes over the longer time period predisposes the cells to apoptosis and may result in a degree of cell shrinkage. In this situation, it must be considered that the control baseline may have changed and the measurement may not be of hypertrophy per se, but of the cytoprotective and anabolic effects of insulin and IGF-1.

## Acknowledgement

This work was funded by the British Heart Foundation.

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