RESEARCH COMMUNICATION

Early phosphoinositide 3-kinase activity is required for late activation of protein kinase C ϵ in platelet-derived-growth-factor-stimulated cells: evidence for signalling across a large temporal gap

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At least two signalling systems have the potential to contribute to the activation of protein kinase C (PKC) family members such as PKC ϵ . One of these is phosphoinositide 3-kinase (PI 3-kinase), whose lipid products activate PKC ϵ *in vitro* and in living cells. The recent observation that there are multiple waves of PI 3-kinase and PKC ϵ activity within the G₀-to-S phase interval provides a new opportunity to investigate the relationship between these two signalling enzymes *in vivo*. We have assessed the relative importance of the early and late waves of PI 3-kinase activity for the corresponding waves of PKC ϵ activity. Blocking the first phase of PI 3-kinase activity inhibited both early and late activation of PKC ϵ . In contrast, the second wave of PI 3-kinase activity was dispensable for late activation of PKC ϵ . These findings suggested that early PI 3-kinase activation induced

a stable change in PKC ϵ , which predisposed it to subsequent activation by lipid cofactors. Indeed, partial proteolysis of PKC ϵ indicated that early activation of PI 3-kinase led to a conformation change in PKC ϵ that persisted as the activity of PKC ϵ cycled. We propose a two-step hypothesis for the activation of PKC ϵ *in vivo*. One step is stable and depends on PI 3-kinase, whereas the other is transient and may depend on the availability of lipid cofactors. Finally, these studies reveal that PI 3-kinase and PKC ϵ are capable of communicating over a relatively long time interval and begin to elucidate the mechanism.

Key words: conformational change, multiple waves of signalling, phosphorylation.

INTRODUCTION

Growth factors such as platelet-derived growth factor (PDGF) trigger cellular responses by engaging intracellular signalling cascades composed of enzymes such as serine/threonine kinases of the protein kinase C (PKC) family [1–3]. The PKC family consists of 12 isoforms that are organized according to primary amino acid sequence, as well as their cofactor requirements for activity, into the classic, novel and atypical groups [4,5]. PKC μ /protein kinase D and protein-related kinases 1–3 are distant family members that do not readily fit into any of the three subgroups.

Exposure of cells to growth factors results in the elevation of lipid cofactors such as diacylglycerol (DAG) and phosphoinositide 3-kinase (PI 3-kinase) lipid products, which activate multiple PKC family members [6]. Enzymes such as PKC ϵ can be activated by both types of lipid cofactors, and hence raises the question of the relative contribution of the two cofactors to PKC ϵ activation.

The finding that PKC ϵ is activated at multiple times in a PDGF-stimulated cell [7] raises an additional question of which lipid cofactors activate PKC ϵ during these two distinct phases of PKC ϵ activity. In PDGF-stimulated cells the first wave of PKC ϵ activity is within 10 min, the activity returns to baseline and then increases again in mid-G₁ phase (5–9 h after stimulation) [7].

Several groups have found that the early activation of PKC ϵ is dependent on PI 3-kinase [8–11]. However, the same did not seem to be true for the late wave of PKC ϵ activity [7]. Even though the late rise in PI 3-kinase products (3–7 h) overlapped with the second wave of PKC ϵ activity (5–9 h), PI 3-kinase inhibitors did not block the second wave of PKC ϵ activation [7,12]. This could be because PKC ϵ is activated by other lipids (such as DAG), and/or that PI 3-kinase plays different roles in the early and late phases of PKC ϵ activation. The goal of the studies described herein was to investigate the relative role of PI 3-kinase for activation of the early versus late phases of PKC ϵ .

MATERIALS AND METHODS

Cell lines and antibodies

HepG2 cells expressing wild-type PDGF β receptors (β PDGFRs), previously described in [13], were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) foetal bovine serum (FBS) and 100 units/ml each of penicillin and streptomycin. Polyclonal antibodies against PKC ϵ and a broad range of actin isoforms were obtained from Santa Cruz Biotechnology (catalogue numbers sc-214 and sc-1615 respectively; Santa Cruz, CA, U.S.A.). Peroxidase-conjugated goat anti-rabbit antibodies were purchased from

Abbreviations used: DAG, diacylglycerol; PDGF, platelet-derived growth factor; β PDGFR, PDGF β receptor; PKC, protein kinase C; PI 3-kinase, phosphoinositide 3-kinase; PP1, protein phosphatase 1; DMEM, Dulbecco's modified Eagle's medium; FBS, foetal bovine serum.

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Calbiochem (La Jolla, CA, U.S.A.) and anti-goat antibodies were purchased from Santa Cruz Biotechnology. Antibodies against β PDGFRs were raised against a glutathione S-transferase fusion protein including the C-terminus [14]. The PKC-selective synthetic peptide (Ac-FKKSFKL-NH₂) used in the PKC kinase assay was described in [15]. Chymotrypsin was purchased from Worthington Biochemical Corporation (Lakewood, NJ, U.S.A.), and wortmannin was purchased from Sigma (St. Louis, MO, U.S.A.), PDGF BB was obtained from R&D Systems (Minneapolis, MN, U.S.A.). Protein phosphatase 1 (PP1) was purchased from New England Biolabs (Beverly, MA, U.S.A.).

Immunoprecipitation

Subconfluent HepG2 cells were serum starved in DMEM plus 0.2% FBS and 0.2 mg/ml BSA at 37 °C and 5% CO₂ for 30 h. PDGF or PDGF buffer was added to the media and incubation was continued for the indicated period of time. Cells were washed twice with ice-cold wash buffer (20 mM Hepes, pH 7.4, 150 mM NaCl) and lysed in lysis buffer [10 mM Tris/HCl, pH 7.4, 5 mM EDTA, 150 mM NaCl, 50 mM NaF, 1% (v/v) Triton X-100, 20 μ g/ml aprotinin, 1 mM PMSF, 1 mM sodium orthovanadate, 10% (v/v) glycerol]. The lysates were centrifuged at 13000 *g* for 15 min at 4 °C and the protein concentration of the clarified lysates was determined using the bicinchoninic acid ('BCA') protein assay (Pierce, Rockford, IL, U.S.A.). Antibodies against PKC ϵ were added to 1 mg of cell lysate. The immune complexes were bound to Protein A-Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.), washed twice with wash buffer (10 mM Pipes, pH 7.0, 100 mM NaCl, 20 μ g/ml aprotinin) plus 0.5% Nonidet P40 and 0.5 M LiCl, twice with wash buffer plus 0.5 M NaCl and twice with wash buffer. Immunoprecipitates were resuspended in 30 μ l of wash buffer and used immediately.

PKC ϵ kinase assay

The PKC ϵ kinase assays were carried out as described in [7]. Briefly, PKC immunoprecipitates were incubated with 150 nM of PKC-selective synthetic peptide (Ac-FKKSFKL-NH₂) in kinase buffer (10 mM Hepes, 1 mM dithiothreitol, 5 μ Ci [γ -³²P]ATP, 10 μ M ATP, 10 mM MnCl₂) for 10 min at 25 °C in a final volume of 80 μ l. The reaction was stopped by centrifugation at 3000 *g* for 5 min, which separated the immobilized PKC immunoprecipitates from the soluble substrate. The supernatant from each of the samples was spotted on to P81 ion-exchange chromatography paper (Whatman Biosystems Ltd., Maidstone, Kent, U.K.), washed four times with 1% (v/v) phosphoric acid and the ³²P incorporation into the peptide was detected by liquid scintillation counting.

Western blotting

Cell lysates were heated at 95 °C in SDS/PAGE buffer [200 mM Tris/HCl, pH 6.8, 10 mM EDTA, 4% (w/v) SDS, 5.6 M 2-mercaptoethanol, 20% glycerol, 0.2% Bromophenol Blue] for 4 min, and resolved by SDS/PAGE and transferred on to Immobilon (Millipore, Bedford, MA, U.S.A.). Membranes were blocked with TBST buffer (10 mM Tris base, pH 8.0, 150 mM NaCl, 0.2% Tween-20) plus 5% (w/v) non-fat dry milk powder for 1 h, probed with primary antibodies, washed with rinse buffer (10 mM Tris base, pH 7.5, 150 mM NaCl) and probed with peroxidase-conjugated secondary antibodies. Proteins were visualized using ECL[®] (Amersham Pharmacia Biotech).

Proteolytic digestion

Cells were stimulated and harvested as described under 'Immunoprecipitation', except PMSF was omitted from the lysis buffer. For PP1 treatment cells were lysed in lysis buffer containing 50 mM Tris/HCl, pH 7.5, 0.1 mM EDTA, 5 mM dithiothreitol, 1 mM MnCl₂, 150 mM NaCl, 1% Triton X-100, 20 μ g/ml aprotinin and 1 mM sodium orthovanadate. A final concentration of 26 μ g/ml chymotrypsin was added to 30 μ g of cell lysate and the proteolysis reaction mixture was incubated for the indicated time period at 30 °C. SDS/PAGE buffer was added to terminate the reaction, and then the samples were resolved by SDS/PAGE and subjected to an anti-PKC ϵ Western blot.

RESULTS AND DISCUSSION

First phase of PI 3-kinase activity is required for both early and late activation of PKC ϵ

To investigate the relative role of PI 3-kinase for activation of the early versus late phases of PKC ϵ , we did the following experiments. Pretreating cells with wortmannin eliminated both the early and late phases of PKC ϵ activity (Figure 1), suggesting that PI 3-kinase was needed at both times. In contrast, the second phase of PKC ϵ activity was unimpeded when only the late wave of PI 3-kinase was blocked (Figure 1). Similar results were also seen when LY294002 was used in place of wortmannin (Figure 1). Note that wortmannin only effectively inhibited PI 3-kinase for approx. 2 h in these cells [12], which is why wortmannin was added in two doses spanning the second wave of PI 3-kinase activity. The data in Figure 1 indicate that early activation of PI 3-kinase is required for both the early and late phases of PKC ϵ activity. We focused our subsequent studies on how early activation of PI 3-kinase was impacting the late wave of PKC ϵ activity.

To explain how PI 3-kinase and PKC ϵ are communicating over the relatively large temporal gap we propose the following

Figure 1 PI 3-kinase activity in early G1 is required for both the first and second phase of PKC ϵ activity

Quiescent HepG2 cells expressing β PDGFRs were stimulated with 50 ng/ml PDGF for the indicated time period. Wortmannin (W) or LY294002 (LY) were added before or after PDGF. When added after PDGF, the wortmannin or LY294002 were administered at 3 h (100 nM and 50 μ M respectively). Wortmannin or LY294002 were added again at 5 h (50 nM and 25 μ M respectively), since the half-life of wortmannin and LY294002 in these cells is approx. 90 min and 3.5 h respectively [12]. Cells were lysed, PKC ϵ was immunoprecipitated and subjected to an *in vitro* kinase assay using a PKC-selective synthetic peptide. The reactions were terminated and then spotted on to P81 paper, washed and the amount of radioactivity was determined by scintillation counting. The data are presented as the fold increase compared with buffer-treated control cells. The results are means $\pm$ S.E.M. for three experiments.

Figure 2 Two-step hypothesis for activation of PKC ϵ *in vivo*

In resting cells PKC ϵ is inactive. PDGF-stimulated PI 3-kinase activity induces a stable change in the conformation of PKC ϵ which enables it to respond to lipids. An increase in the availability of lipid cofactors [DAG or PI 3-kinase (PI3K) lipid products] fully activates PKC ϵ . Loss of these lipid cofactors results in the rapid reversion of active PKC ϵ to the PKC ϵ * state.

Figure 3 PKC ϵ is a long-lived protein

Cells were or were not pretreated with 2 μ M cycloheximide, and then either left resting or exposed to 50 ng/ml PDGF for the indicated time period. The cells were then lysed and 30 μ g of protein was resolved by SDS/PAGE and subjected to Western blotting analysis using antibodies against PKC ϵ , actin or β PDGFR.

two-step hypothesis (Figure 2). The first step is dependent on early activation of PI 3-kinase, and leads to a stable change in PKC ϵ . This change persists even when the levels of PI 3-kinase products and PKC ϵ activity recede to basal levels. The second step involves the accumulation of lipid cofactors such as PI 3-kinase products or DAG and activates the enzyme. While the second step of the hypothesis, that lipid cofactors activate PKC ϵ , has been well established, the novel component of the hypothesis is that PKC ϵ undergoes a conformational change that is stable, PI 3-kinase-dependent, and independent of the changes in PKC ϵ activity.

PKC ϵ is a long-lived protein

If the hypothesis in Figure 2 is correct, then PKC ϵ should be a long-lived protein. To investigate this issue, we examined the turnover of PKC ϵ . Cycloheximide blocks protein synthesis and leads to a loss of proteins with a relatively short half-life. For instance, the β PDGFR has a half-life of approx. 30 min in PDGF-stimulated cells [16], and the vast majority of the receptor disappeared within 1 h in cycloheximide-treated cells (Figure 3). In contrast, long-lived proteins such as actin did not change appreciably within the time frame of the experiment. Similarly, there was no change in the amount of PKC ϵ under these

Figure 4 Susceptibility of PKC ϵ to proteolysis before and after PDGF stimulation

Upper panel: cells were stimulated with 50 ng/ml PDGF for the indicated period of time, lysed, chymotrypsin was added to the lysates and the resulting samples were incubated at 30 °C. SDS/PAGE buffer was added to terminate the reaction, and the proteins were resolved by SDS/PAGE and subjected to Western blotting analysis using an anti-PKC ϵ or an anti-actin antibody. The indication of PKC ϵ activity included at the very top of the Figure is taken from Figure 1, and is included to underscore the lack of correlation between protease sensitivity and enzymic activity. 15', 15 min etc. Lower panel: the amount of PKC ϵ was quantified densitometrically and plotted as a percentage of the unproteolyzed PKC ϵ . □, - PDGF; ◇, + PDGF (10 min); ○, + PDGF (1 h); △, + PDGF (7 h).

conditions (Figure 3), indicating that the molecules of PKC ϵ activated during the first wave were likely to persist throughout the second phase of PKC ϵ activity.

PDGF stimulation increases the susceptibility of PKC ϵ to partial proteolysis

To test the hypothesis that the activation of PKC ϵ involves a conformational change we tested its susceptibility to proteolysis by adding chymotrypsin to lysates prepared from resting or stimulated cells. Indeed, PKC ϵ in lysates made from PDGF-stimulated cells was more rapidly proteolysed than in unstimulated cells (Figure 4). Interestingly, this increased sensitivity to proteolysis persisted even when PKC ϵ activity receded (1 h time point) or was re-elevated (7 h time point; Figure 1). Hence, PDGF increased the susceptibility of PKC ϵ to proteolysis, suggesting that the conformation of the enzyme had changed. Furthermore, this was a stable change, and it persisted as cells moved through G₁.

Since binding of the lipid cofactors to PKC ϵ is likely to induce a conformational change, we expected to see a difference in the proteolytic sensitivity of PKC ϵ at the times when PI 3-kinase lipids were elevated (at 10 min and 7 h) compared with when the lipid levels were low (at 1 h) [12]. However, this is not what we observed. The enhanced sensitivity to proteolysis was observed regardless of the cellular levels of PI 3-kinase products, which suggests that the change in proteolytic sensitivity did not reflect binding to PI 3-kinase lipids. We tried two other proteases, (trypsin and proteinase K), which proteolysed PKC ϵ , but we did

Figure 5 First wave of PI 3-kinase activity is required to increase PKC ϵ sensitivity to proteolysis

Upper panel: cells were stimulated with 50 ng/ml PDGF for 7 h, harvested and the resulting samples were analysed exactly as in Figure 4, except that some of the cells were treated with wortmannin. The Western blot of actin was included as an indication of protein loaded in each lane. 15', 15 min etc. Lower panel: the amount of PKC ϵ was quantified densitometrically and plotted as a percentage of the unproteolyzed PKC ϵ . $\square$, — PDGF; $\diamond$, + PDGF (wortmannin added 10 min before PDGF); $\circ$, + PDGF (wortmannin added 3 and 5 h after PDGF).

not find any difference between PKC ϵ at the time points tested. It is likely that the partial proteolysis approach is not sufficiently sensitive to detect changes that may be induced by the binding of lipid cofactors, or alternatively, we had not chosen the correct conditions to reveal such differences.

While a change in the proteolytic sensitivity of a protein is an indicator of a conformational change, we fully appreciate that our findings do not prove that PKC ϵ is undergoing a conformational change. It is possible that PDGF is changing the composition and/or localization of the PKC ϵ binding partners, which alters the availability of proteolytic sites in PKC ϵ . The use of purified proteins and *in vitro* systems that accurately mimic PKC ϵ activation will be necessary to resolve these possibilities. Our findings reveal a PDGF-induced stable change in the sensitivity of PKC ϵ to partial proteolysis.

First wave of PI 3-kinase activity is required to increase PKC ϵ sensitivity to proteolysis

The hypothesis in Figure 2 predicts that only the first phase of PI 3-kinase activity contributes to the stable change in PKC ϵ conformation. To test this prediction the early and late phases of PI 3-kinase activity were blocked with wortmannin and the susceptibility of PKC ϵ to proteolysis was tested. Figure 5 shows that inhibiting the first wave of PI 3-kinase activity largely eliminated the increased sensitivity of PKC ϵ to proteolysis. In contrast, blocking the second wave of PI 3-kinase activity had only a minor effect. These results indicate that the first, but not the second, wave of PI 3-kinase activity is involved in inducing a conformational change in PKC ϵ . Furthermore, they indicate that PI 3-kinase is essential for this PDGF-dependent effect on PKC ϵ .

Figure 6 Susceptibility of PKC ϵ to proteolysis is phosphorylation dependent

Cells were left resting or stimulated with PDGF for 10 min, lysed and the resulting lysates were incubated at 30 °C without any additions (lanes 1–4), 1 unit of PP1 (lanes 5–8) or 1 unit of PP1 and 10 mM sodium orthovanadate (lanes 9–12). After this time 10 mM sodium orthovanadate was added to all of the samples, as well as 26 μ g of chymotrypsin. The incubation at 30 °C was continued for 60 min, and then the samples were analysed by Western blotting using antibodies directed against PKC ϵ or actin. The actin Western blot verified that comparable amounts of cell lysate were present in all of the lanes.

Our findings indicate that PI 3-kinase makes both a direct and indirect effect on the activation of PKC ϵ . The direct effect is when PI 3-kinase products serve as lipid cofactors necessary for the activation of PKC ϵ , and this issue has been well documented in the past. The novelty of this report is the identification and partial resolution of an indirect effect of PI 3-kinase on PKC ϵ . The effect is indirect because it lasts even when PI 3-kinase products are no longer elevated.

We have begun to investigate the molecular nature of the indirect effect. The subcellular location of PKC ϵ did not change detectably in response to PDGF, whereas PMA induced a stoichiometric redistribution of PKC ϵ to the particulate fraction (results not shown). Phosphorylation is essential for the maturation of newly synthesized PKC family members [15,17], and at least some of these kinases are regulated by PI 3-kinase (e.g. phosphoinositide-dependent kinase 1). Consequently, we tested the idea that phosphorylation of PKC ϵ was involved with changing its proteolytic sensitivity. To this end we pretreated cell lysates prepared from resting or PDGF-stimulated cells with PP1, and then tested the sensitivity of PKC ϵ to proteolysis. As shown in Figure 6, PP1 reversed the PDGF-dependent increase of PKC ϵ to chymotrypsin (compare lanes 2 and 4 with lanes 6 and 8). This phenomenon was dependent on the phosphatase activity of PP1, since PP1 had no effect on PKC ϵ proteolysis when it was added together with sodium orthovanadate (lanes 9–12). These data suggest that the PDGF-dependent change in the sensitivity of PKC ϵ to proteolysis involved phosphorylation. We tried to extend these studies using phosphospecific antibodies. However, we failed to find PDGF-dependent changes in the phosphorylation state of the residues in the activation loop (Thr⁵⁶⁶), or the turn (Thr⁷¹⁰) and hydrophobic (Ser⁷²⁹) regions of the C-terminus (E. Balciunaite and A. Kazlauskas, unpublished work). Whether this relates to the quality of the phosphospecific reagents that we tried, or accurately indicates that there were no changes in the phosphorylation state at any of these sites, we are unable to determine at this point.

One caveat of the proposed two-step hypothesis is that it does not account for how phorbol esters activate PKC ϵ , which does not involve an obvious PI 3-kinase-dependent step. One possibility is that reagents such as PMA or a large bolus of exogenously added DAG do not accurately reflect the physiological sequence of events by which PKC ϵ is activated. Alternatively, there are multiple ways in which PKC ϵ can be activated, and the studies presented herein are most relevant to the sequence of events occurring in growth-factor-stimulated cells.

The finding that early signalling events (activation of PI 3-kinase) can stably alter signalling enzymes (PKC ϵ) in a way that predisposes them to activation several hours later reveals the ability of signalling enzymes to communicate across relatively long periods of time. In addition, these findings reinforce the concept that there are important signalling events well beyond the first 30 min of signalling. Indeed, numerous laboratories have demonstrated that driving a cell from G₀ into S phase requires signalling enzymes in mid or late G₁ [7,12,18–21]. Further investigation of the prolonged second wave of signalling is likely to enhance our understanding of how signalling engages cellular responses such as progression through the cell cycle. In addition, this system will continue to provide an excellent setting in which to study how signalling enzymes communicate over large temporal gaps.

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