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PKCα reduces the lipid kinase activity of the p110α/p85α PI3K through the phosphorylation of the catalytic subunit

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Abstract

The modulation of phosphoinositide 3-kinase (PI3K) activity influences the quality of cellular responses triggered by various receptor tyrosine kinases. Protein kinase C (PKC) has been reported to phosphorylate signalling molecules upstream of PI3K and thereby it may affect the activation of PI3K. Here, we provide the first evidence for a direct effect of a PKC isoenzyme on the activity of PI3K. PKCα but not PKCε phosphorylated the catalytic subunit of the p110α/p85α PI3K in vitro in a manner inhibited by the PKC inhibitor bisindolylmaleimide I (BIM I). The incubation of PI3K with active PKCα resulted in a significant decrease in its lipid kinase activity and this effect was also attenuated by BIM I. We conclude that PKCα is able to modulate negatively the lipid kinase activity of the p110α/p85α PI3K through the phosphorylation of the catalytic subunit.

Keywords: PI3K; PKC; Isoenzymes; Isoenzyme-specific functions; Signal transduction

Upon the stimulation of receptor tyrosine kinases autophosphorylation produces phosphotyrosine containing docking sites which bind and lead to the activation amongst other effectors phosphoinositide 3-kinase (PI3K) [1,2]. In response to growth factors and hormones, the PI3K catalyses the phosphorylation of phosphatidylinositol lipids at the D-3 position of the inositol ring and thereby initiates a coordinated set of events that control cell survival, cell growth, migration or metabolic changes [3]. Various signalling proteins, such as exchange factors that regulate small GTPases, serine/threonine kinases, and tyrosine kinases, contain domains that specifically bind to PI3K products [3,4]. Upon PI3K activation these proteins accumulate beneath the plasma membrane through interaction with the newly formed phosphoinositides. Consequent to this translocation effectors are activated by one of a number of means triggering several responses, such as assembly of signalling complexes, branching polymerisation of actin, and priming of kinase cascades [5]. Alterations in the fine-tuning of PI3K activity may be responsible for pathological processes since this pathway is hyperactivated in some cancers, and defects in the pathway contribute to type II diabetes [3,6–8].

Hepatocyte growth factor/scatter factor (HGF) is an established strong activator of PI3K and induces growth in certain cell types and scattering in others [9,10]. The spatio-temporal regulation of the signalling routes through PI3K might at least partially be responsible for the variations in cellular responses triggered by HGF. We have studied the signalling pathways that lead to the HGF-induced migration of HepG2 human hepatoma cells [11] and have found that PKC modulates negatively the HGF-induced motility of these cells and the duration of PI3K activation [12]. To date, PKCs have been thought to be regulated by PI3K through 3-phosphoinositide-dependent protein kinase-1 (PDK1) phosphorylation that is necessary for the maturation of certain PKC isoenzymes [13,14]. On the other hand, PKCs are also able to phosphorylate various signalling molecules upstream of PI3K including receptors and docking proteins, and

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thereby to influence the activation of PI3K [15–19]. However, in the context of HGF response, PKC had no effect on the tyrosine phosphorylation of the signalling proteins upstream of PI3K in HepG2 cells [12,20]. In addition, the basal activity of PI3K decreased rapidly in cells treated with a direct PKC activator [12]. These observations led to the idea that PKC controls PI3K. Recent studies have provided evidence that there is a PKC control acting on the HGF stimulated ERK pathway that is determined by location [20,21]. For PI3K there is no evidence that location/recruitment is modified by PKC (with respect to receptor, Gab1 tyrosine phosphorylation), hence the effect of PKC may be direct. This hypothesis is tested here and evidence presented that PI3K (the p110α subunit) is a direct substrate for PKCα in vitro affecting its catalytic activity.

Materials and methods

Materials. Human, recombinant, Sf21 cells-expressed, purified PKCα (Cat. No. 14-484), PKCε (Cat. No. 14-518), and PI3K p110α/p85α (Cat. No. 14-602) were from Upstate cell signaling solutions, Lake Placid, NY, USA. Phorbol myristate acetate (PMA), L-α-phosphatidylinositol, and L-α-phosphatidylserine were obtained from Sigma–Aldrich. The PKC inhibitor BIM I (also known as GF 109203X) and the PI3K inhibitor LY294002 were the products of Calbiochem. All the other chemicals used were of the highest grade available.

Preparation of PKC activator micelles. L-α-Phosphatidylserine (47 μL of 10 mg/mL) was air-dried in a glass tube, resuspended with 10 μL of 10% Triton X-100, 40 μL of 20 mM Tris, pH 7.4, and 0.5 μL of 1.6 mM PMA. The suspension was vortexed 6 times for 10 s to form mixed detergent micelles.

In vitro phosphorylation of PI3K with PKC. For each reaction 500 ng p110α/p85α PI3K was incubated in the presence or absence of 250 ng PKC in 30 μL of 20 mM Tris buffer, pH 7.4, containing 6 μL micelles, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 20 μM ATP, and 2 μCi [γ-^{32}P]ATP at 30 °C in a thermomixer for the times indicated. Where used, BIM I and LY294002 were at 1 and 5 μM concentrations, respectively. The reactions were stopped with the addition of 10 μL Laemmli sample buffer supplemented with 10 mM EDTA. The samples were boiled for 5 min and subjected to SDS–PAGE in 7.5% gels. The gels were Coomassie-stained, dried, and subjected to autoradiography for 12 h. For the calculation of phosphorylation stoichiometry the radiolabelled bands were excised from the gels and the proteins were solubilised in 5 N NaOH and after the neutralisation of the solutions the radioactivity was measured based on the Cerenkov effect in a liquid scintillation spectrometer.

PI3K kinase assay. For the measurement of lipid kinase activity, PI3K was pre-incubated in the presence or absence of PKC as described above, but with non-radiolabelled ATP. Five microlitre aliquots of these pre-incubation mixtures were used for each lipid kinase activity assay. The PI3K activity was measured in duplicate as incubating 20 μg L-α-phosphatidylinositol with the pre-incubated PI3K sample in 50 μL 20 mM Tris, pH 7.4, buffer containing 5 mM $MgCl_2$, 100 mM NaCl, 20 μM ATP, and 2 μCi [γ-^{32}P]ATP at 30 °C in a thermomixer for 15 min. The reactions were stopped with the addition of 50 μL of 2 N HCl, and the lipids were extracted with 200 μL chloroform/methanol 1/1. The phases were separated by centrifugation at 4 °C for 5 min, the lower chloroform phase was concentrated and then spotted onto a silica gel TLC plate that had been pre-activated with 1% K-oxalate, 2 mM EDTA, and 0.5 M boric acid. The TLC plates were run in chloroform/methanol/water/ammonia 90/70/14.6/5.4 and subjected to autoradiography for 2 h. In some assays, the PI3K activity was measured in triplicate, 1 μCi ^{32}P-labelled ATP was used in the assay buffer per reaction, and the radioactivity incorporated into lipids was counted directly as the separated chloroform phase was mixed into toluene liquid scintillation cocktail. In the experiments where the phosphotransferase activity of PKC was inhibited with BIM I, during the pre-incubation period, the PI3K assay buffer was also supplemented with 1 μM BIM I.

Results and discussion

PKCα but not PKCε phosphorylates the p110α subunit of PI3K in vitro

Both subunits of PI3K purified from mammalian tissues have been reported to be highly phosphorylated [22]. The autophosphorylation of the p85α regulatory subunit on Ser608 is known to reduce the lipid kinase activity of the functional p110α/p85α PI3K heterodimer [23]. However, little has been known about the regulation of PI3K activity by other Ser/Thr phosphorylations of either subunits.

Using human, recombinant, purified enzymes in in vitro assays the autophosphorylation of PI3K on its p85α regulatory subunit was confirmed in our experiments. Notably however, we have found that in the presence of PKCα the p110α catalytic subunit of PI3K became phosphorylated (Fig. 1). This response displayed specificity as in parallel experiments PKCε efficiently autophosphorylated itself, but failed to catalyse the phosphorylation of the p110α PI3K subunit. The PKC inhibitor BIM I inhibited the autophosphorylation of the PKC isoforms and reduced strongly the trans phosphorylation of the p110α PI3K subunit that was observed (Fig. 1A). This indicates that the effect of PKCα is indeed catalytic. Though the autophosphorylation of PI3K seemed to be decreased in the presence of PKCε, this effect was not inhibited by BIM I, hence it was not due to the catalytic activity of PKCε. Consistent with PKCα acting catalytically rather than allosterically on p110α, the PI3K inhibitor LY294002 decreased the autophosphorylation of PI3K on the p85α subunit as expected, but had no effect on the phosphorylation of the p110α subunit catalysed by PKCα (Fig. 1B).

The stoichiometry of PI3K autophosphorylation and the PKCα-catalysed phosphorylation of the p110α were comparable. Under our experimental conditions, the incorporations of phosphate to proteins were 0.35 ± 0.05 mol/mol for PI3K autophosphorylation and 0.22 ± 0.03 mol/mol for the PKCα-catalysed phosphorylation of p110α. However, after longer incubation the autophosphorylation of the regulatory subunit reached a higher stoichiometry than the phosphorylation of the catalytic subunit (the maximal values were 0.65 and 0.3 mol/mol, respectively). The possibility that these sites were already partially occupied cannot be excluded since the baculovirus-expressed PI3K has been reported to become more active upon phosphatase treatment [24].

These results indicate that of the two isoenzymes tested PKCα but not PKCε is able to phosphorylate the p110α subunit of PI3K in vitro. Interestingly, an earlier report demonstrated that upon triggering the T-cell antigen receptor in T-cells or activation of PKC directly with phorbol ester treatment, the p110 subunit associated with the p85α subunit of PI3K became phosphorylated exclusively

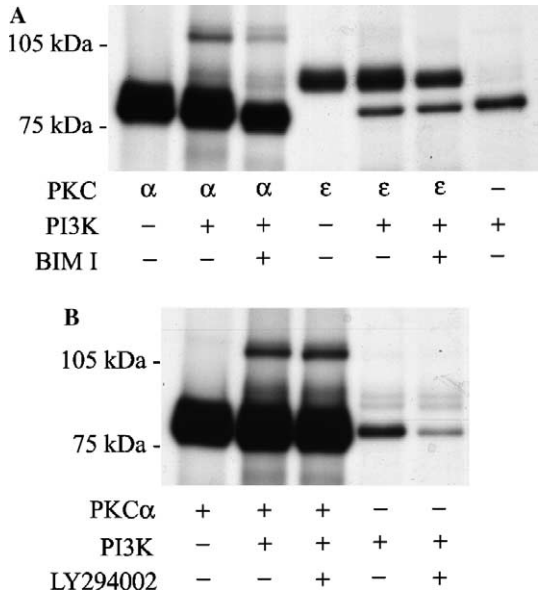

Fig. 1. PKCα but not PKCε phosphorylates the catalytic subunit of the p110α/p85α PI3K. (A) The autophosphorylation of PKCα (lanes 1–3, 80 kDa), of PKCε (lanes 4–6, 90 kDa), and of PI3K on the regulatory subunit (lane 7, 85 kDa) can be observed. The catalytic subunit of PI3K is phosphorylated in the presence of PKCα (lanes 2 and 3, 110 kDa), and this phosphorylation is substantially suppressed by the PKC inhibitor BIM I (lane 3, the autophosphorylation of PKCα is also decreased). PKCε is not able to phosphorylate the PI3K catalytic subunit (lanes 5 and 6, no 110 kDa signal). The 85 kDa signal in the presence of PKCε (lanes 5–6) is due to the autophosphorylation of PI3K on the regulatory subunit since it is not increased compared to PI3K alone (lane 7) and is not influenced by BIM I (lane 6). The autophosphorylation of the PI3K regulatory subunit is covered by the intensive signal of the autophosphorylated PKCα in lanes 2 and 3. (B) The phosphorylation of the PI3K catalytic subunit observed in the presence of PKCα (lanes 2 and 3, 110 kDa) is not influenced by the PI3K inhibitor LY294002 (lane 3). LY294002 efficiently inhibits the autophosphorylation of PI3K on the regulatory subunit (lane 5). Human, recombinant, purified PKCα, PKCε, and p110α/p85α PI3K were used in in vitro kinase assays for 30 min as described in detail under Materials and methods. The proteins were separated in 7.5% SDS–PAGE and subjected to autoradiography for 6 h. The panels show representatives of 3-3 independent experiments, respectively.

on serine [25]. Our data indicate that this T-cell response likely reflects direct phosphorylation of PI3K by PKC and further that this may represent part of a feedback control on receptor stimulated PI3K activity (see below).

PKCα phosphorylation of the p110α subunit reduces PI3K lipid kinase activity

We investigated whether the PKCα-catalysed phosphorylation of the p110α subunit had any effect on the lipid kinase activity of the functional p110α/p85α PI3K heterodimer. When the p110α/p85α PI3K was pre-phosphorylated with PKCα it produced far less phosphatidylinositol-3-phosphate (PI3P) from phosphatidylinositol (PI) than the enzyme pre-incubated without PKCα. This is clearly illustrated in Fig. 2 showing the TLC separation of the chloroform extracted lipids. In subsequent experiments, we measured the activity of PI3K by directly determining the radioactivity incorporated

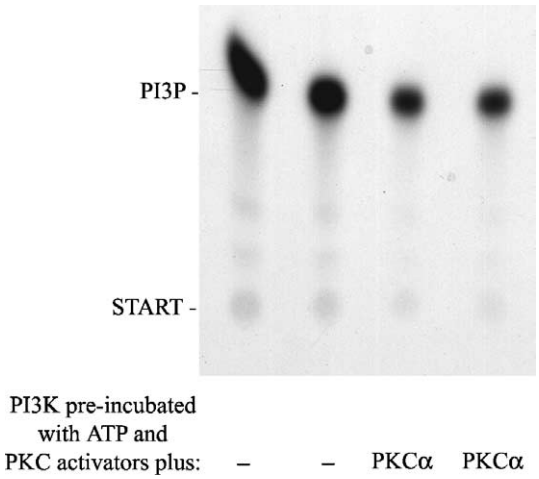

Fig. 2. The PKCα-phosphorylated p110α/p85α PI3K has lower lipid kinase activity. PI3K was pre-incubated with ATP and PKC activators in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of PKCα for 60 min. Aliquots of the protein phosphorylation mixtures containing equal amounts of PI3K were used in in vitro lipid kinase assays on phosphatidylinositol for 15 min. The lipids were separated by TLC and subjected to autoradiography for 2 h. The experiments were carried out as described in detail under Materials and methods. A representative of three independent experiments is shown.

into lipids, since radioactivity from ^{32}P-labelled ATP was incorporated almost exclusively to PI3P (Fig. 2). The PKCα phosphorylation reduced the activity of PI3K significantly with the activity being reduced to 50% of the control (Fig. 3). When the phosphotransferase activity of PKCα was inhibited with BIM I the PKC-induced reduction of PI3K activity was considerably attenuated. The rate of the PKCα-catalysed phosphorylation of the p110 catalytic

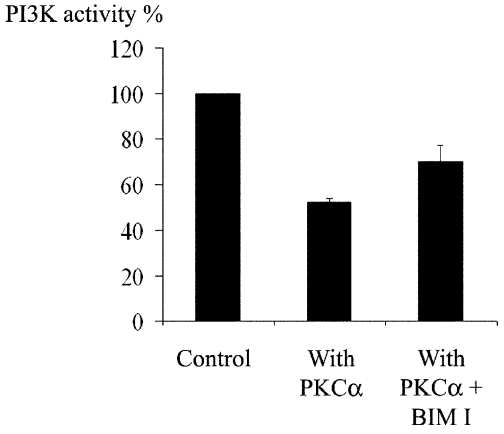

Fig. 3. Phosphorylation by PKCα reduces the lipid kinase activity of PI3K. PI3K was pre-incubated with ATP and PKC activators in the absence or presence of PKCα for 60 min. The catalytic function of PKCα was inhibited with BIM I. Aliquots of the protein phosphorylation mixtures containing equal amounts of PI3K were used in in vitro lipid kinase assays on phosphatidylinositol for 15 min. The experiments were carried out using triplicate samples as described in detail under Materials and methods. The PI3K activity was calculated from the radioactivity incorporated into lipids. The relative activities shown by the columns were calculated within the same individual experiments. The mean values obtained from the results of three independent experiments are shown.

subunit and the rate of the BIM I-dependent decrease in PI3K activity correlated well. These data show that the PKCα-catalysed phosphorylation of the p110α subunit modulates negatively the lipid kinase activity of the functional p110α/p85α PI3K heterodimer.

The current study reveals the first example of a new mechanism that can regulate the activity of PI3K, and explains the results we and others published previously in cellular studies. Based on these data, we conclude that there is a direct link between PKC and PI3K, the lipid kinase activity of PI3K being decreased by PKCα directly through phosphorylation in an isoenzyme-specific manner. Various receptors have differing abilities to activate certain PKC isoenzymes and hence differing potentials for implementing this feedback control loop are predicted. The regulatory mechanism we have identified is likely to have significance in different physiological processes, almost prominently in those systems where the intensive activation of PI3K has a central role. In the signalling system of HGF which triggers growth or migration, the activation of PKCα may provide the negative feedback control linked into the dynamic signals associated with migration.

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