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Katja Lehmann, Jörg P Müller, Bernhard Schlott, Philipp Skroblin, Dagmar Barz, et al.. PI3K γ controls oxidative burst in neutrophils via interaction with PKC α and p47phox. *Biochemical Journal*, 2009, 419 (3), pp.603-610. 10.1042/BJ20081268 . hal-00479057

HAL Id: hal-00479057

<https://hal.science/hal-00479057>

Submitted on 30 Apr 2010

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PI3K γ controls oxidative bursts in neutrophils via interactions with PKC α and p47phox

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Key words: PI3K γ , PKC α , interaction, protein kinase, reactive oxygen species, neutrophils.

page heading title: PI3K γ PKC α interaction

KL and JPM contributed equally

Abbreviations: ROS, reactive oxygen species; PI3K γ , phosphoinositide 3-kinase γ ; PKC α , protein kinase C α ; fMLP, N-formyl-Met-Leu-Phe; BIM, bisindolylmaleimide; PVDF, poly vinylidene difluoride

SYNOPSIS

Neutrophils release reactive oxygen species (ROS) as part of the innate inflammatory immune response. Phosphoinositide 3-kinase γ (PI3K γ), which is induced by the bacterial peptide N-formyl-Met-Leu-Phe (fMLP), has been identified as an essential intracellular mediator of ROS production. However, the complex signalling reactions that link PI3K γ with ROS synthesis by NADH oxidase have not yet been described in detail. We found that activation of neutrophils by fMLP triggers the association of PI3K γ with protein kinase C α (PKC α). Specific inhibition of PI3K γ suppresses fMLP-mediated activation of PKC α activity and ROS production, suggesting that the protein kinase activity of PI3K γ is involved. Our data suggest that the direct interaction of PI3K γ with PKC α forms a discrete regulatory module of fMLP-dependent ROS production in neutrophils.

INTRODUCTION

Neutrophils are key mediators of innate immunity and host defense. After exposure to chemotaxins, freely circulating neutrophils adhere to endothelial cells and migrate into infected tissue. Once they have reached the inflamed area, the neutrophils begin to phagocytize the pathogens. Secretion of reactive oxygen species (ROS) and lysosomal enzymes contributes to the killing of bacteria. Neutrophil defense reactions are tightly controlled by inflammatory mediators, including chemokines, bacterial peptides, and complement fragments.

Bacterial cell wall-derived chemotactic peptides, such as N-formyl-Met-Leu-Phe (fMLP) or complement fragment C5a, are prototypical peptides that affect the cell responses of neutrophils by binding to specific G protein-coupled receptors [1]. The interactions of these chemotaxins with their receptors trigger multiple intracellular signalling pathways, including rapid stimulation of phosphoinositide 3-kinase γ (PI3K γ) [2,3]. This signalling protein is critically involved in the control of neutrophil migration and ROS secretion induced by fMLP and chemokines [4–6]. In neutrophils and other cell types, PI3K γ exists in a heterodimeric form associated with the adapter protein p101. p101 strongly enhances G protein-dependent stimulation of the lipid kinase activity of PI3K γ *in vitro* [7]. Comparative investigations have revealed a substantial decrease in PI3K γ lipid kinase activity and phosphatidylinositol-3,4,5-triphosphate production provoked by chemoattractants in p101^{-/-} neutrophils in comparison with wild-type littermates. In addition, simultaneous loss of chemoattractant-dependent migration has been observed in p101-deficient neutrophils. In compelling contrast with these findings, p101^{-/-} neutrophils exhibit an unaltered oxidative burst induced by fMLP or C5a [8]. These intriguing data point to differential functions for p101 in migration and chemoattractant-induced ROS production of neutrophils. Whereas migration of neutrophils requires interactions between p101 and PI3K γ , ROS production induced by chemoattractants appears to be independent of p101. Consequently, the distinct regulatory effects of PI3K γ on both inflammatory reactions of neutrophils become apparent.

Our current study provides, to our knowledge, the first evidence of a PI3K γ -dependent signalling path for ROS production that does not involve the lipid kinase activity of the signalling protein. Our data reveal the interactions of PI3K γ with PKC α and p47phox in human neutrophils induced by fMLP. Chemoattractant-induced binding of PI3K γ to PKC α coincides with ROS production and is accompanied by a significant increase in PKC α activity. The functional relevance of the proposed PI3K γ /PKC α signalling path will be discussed.

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MATERIALS AND METHODS

Materials

Chemoattractant N-formyl-Met-Leu-Phe (fMLP), PKC inhibitor bisindolylmaleimide I (BIM), bis-N-methylacridinium nitrate (lucigenin), histone type H1 calf thymus (SIGMA, H4524), histopaque 1077, and histopaque 1119 were from Sigma-Aldrich (Taufkirchen, Germany). PI3K γ inhibitor AS605240 was from Alexis; monoclonal mouse PKC α antibody for immunoprecipitation was from BD Biosciences (Heidelberg, Germany); polyclonal rabbit PKC α for immunodetection and polyclonal rabbit p47phox antibody were from Santa Cruz Biotechnology (Santa Cruz, CA); [γ - 32 P]-ATP was from Hartmann Analytik GmbH (Braunschweig, Germany); and monoclonal mouse PI3K γ antibody H1 [8] used for immunodetection was from Jena Bioscience (Jena, Germany). Sequencing-grade modified trypsin was purchased from Promega (Mannheim, Germany). The HEK293 cell line stably expressing the catalytic subunit p110 γ was from our laboratory collection.

Recombinant proteins

For recombinant protein production, the PKC α -encoding gene was amplified by PCR using primers PKC-FW, containing an *Eco*RI restriction site (5'-GCGAATTCGGCATGGCTGACGTTTTCCCGGGC-3'), and PKC α -REV, containing an *Xba*I restriction site (5'-TGCTCTAGAATCATACTGCACTCTGTAAGATGGGG-3'). The amplified gene was subsequently subcloned into plasmid pcDNA [9]. In parallel, a DNA region encoding the GST epitope was amplified using primer GST-FW, containing a *Bam*HI restriction site (5'-CGGGATCCGGCATGTCCCCTATACTAGG-3'), and GST-REV, containing an *Eco*RI restriction site (5'-GAGAATTCAGAACGCGGAACCAGATCCGA-3'). To produce GST-PKC α , the subcloned DNA fragments were fused and inserted into pFastBac1 (Invitrogen, Karlsruhe, Germany). The resulting plasmid pFastBac1-GST-PKC α was chemically transformed into *E. coli* DH10BacTM cells and integrated by site-specific recombination into bacmids containing the host DNA. GST-PKC α -Bacmid was transfected

into SF9 cells, and baculoviruses were produced in accordance with the manufacturer's instructions. The baculovirus SF9 system was used to produce the recombinant proteins PKC α , GST-PKC α , and His-tagged wild-type PI3K γ or catalytically inactive PI3K γ -KR [10], as described previously [11]. Recombinant p47phox and p67phox proteins were produced in *E. coli* as described previously [12]. GST-tagged proteins were purified by affinity chromatography on glutathione-agarose. Subsequently, the GST epitope was cleaved using thrombin.

Affinity precipitation of recombinant proteins

Purified recombinant proteins (1 μ g each) were incubated in kinase buffer for 30 min at 30 °C. Subsequently, epitope-labeled proteins were purified using affinity purification. His-PI3K γ was purified using Ni-NTA-agarose and GST-PKC α was purified using glutathione beads. Affinity chromatography material (50 μ l slurry) was incubated for 1 h with ongoing agitation. Beads were washed extensively at least four times. For purification of His-PI3K γ , imidazole (20 mM) was added to the buffer to reduce nonspecific binding. Affinity-purified material was boiled in protein loading buffer and directly subjected to SDS-PAGE.

Kinase assays

Protein kinase assays using purified His-PI3K γ proteins and GST-PKC α protein were carried out as described in Stoyanova et al. at 30 °C [10]. As indicated, TPA (300 nM), BIM (100 nM), wortmannin (100 nM), or liposomes were added to the reaction mixture. Lipids (100 mM total lipid) were composed of diacylglycerol:phosphatidylinositol-3,4,5-triphosphate:phosphatidylserine:phosphatidylcholine (5:10:40:45 molecular ratio). Specific PKC α activity was determined using calf thymus histone H1 or p47phox as a substrate (total amount, 0.1 μ g/ml) in presence of 5 μ Ci [γ -³²P]-ATP and incubating at 30 °C. In vivo activity of PKC α was determined from freshly prepared neutrophils. PKC α was immunoprecipitated from cell lysates of 3 x 10⁷ cells and subsequently used for kinase assays. Reaction mixtures (25 μ l) were subjected to SDS-PAGE on 12.5 % PAA gels. After electrophoresis, the gels

were washed three times to remove unincorporated ATP and subsequently exposed to P-image analysis. The amount of incorporated ^{32}P was quantified using a phospho-imaging system (FLA500, Fuji, Raytest) and AIDA image analysis software. Differences were considered significant when $P < 0.05$.

Mass spectrometric analysis

Protein gels were stained with Coomassie brilliant blue R250 (Sigma) and appropriate gel sections were subjected to in-gel digestion with trypsin as described by Shevchenko et al. [13] with one modification: reduction and oxidation of thiol groups was performed with a mixture of 0.1 mM tributylphosphine (Sigma-Aldrich, Taufkirchen, Germany) and 0.82 mM 4-vinylpyridine (Aldrich). The trypsin in-gel protein digests were analyzed with LC-ESI-MS equipment consisting of an Ettan micro LC-HPLC (GE Healthcare, Munich, Germany) online coupled to a Finnigan LTQ mass spectrometer (Thermo Electron Corporation, Erlangen, Germany). The HPLC was equipped with a Zorbax 300SB 5 μM , 5 \times 0.3 mm trapping column and a Zorbax 300SB 5 μM , 150 \times 0.075 mm separation column (Agilent, Böblingen, Germany). The separation of peptides on the HPLC occurred by applying a linear gradient running from 0% to 47% acetonitrile, followed by a stepwise elution with 84% acetonitrile in 0.1% formic acid, each under control of Unicorn software (GE Healthcare). The LTQ was operated under control of Xcalibur 1.4 software (Thermo Electron Corporation, Erlangen, Germany). For processing of the CID spectra (MS/MS-spectra) and final protein identification, BioWorks 3.2 software (Thermo Electron Corp., Erlangen, Germany) and the NCBI human protein database were used. Site-specific phosphorylation analysis was performed by scanning pSer and pThr for loss of phosphoric acid (neutral loss of 98) in the respective MS/MS-spectra.

Preparation of neutrophils

Human neutrophils were isolated from peripheral blood from healthy volunteers. The erythrocytes were removed by dextran sedimentation, and the samples were then loaded on

a Histopaque gradient to exclude lymphocytes, monocytes, and platelets. Any remaining erythrocytes were removed by hypotonic lysis in distilled water. Finally, the cells were washed twice with PBS and resuspended in the appropriate buffers. Trypan blue staining revealed that at least 95% of the cells were neutrophils with greater than 95% viability.

Neutrophil stimulation and treatment with inhibitors

Freshly prepared neutrophils were incubated at 37 °C in resuspension buffer (30 mM HEPES, 110 mM NaCl, 10 mM KCl, 1 mM MgCl₂, 1.5 mM CaCl₂, 10% glycerol, and 0.5 mg/ml BSA). If indicated, inhibitors (10 μM AS605240 and 1 μM BIM) were added 30 min before stimulation. Cells were stimulated with 1 μM fMLP for the indicated time periods at 37 °C and stopped by rapid, short centrifugation at 4 °C.

Preparation of membrane and cytosolic fractions

Human neutrophils were isolated as described, aliquoted (3×10^7 cells) in resuspension buffer and stimulated. Cells were then rebuffered in fractionation buffer (25 mM HEPES, 25 mM β-glycerophosphat, 2 mM EDTA, 0.5 mg/ml BSA, 1 mM Na₃VO₄, 1 mM PMSF, 21 μg/ml Aprotinin, 5 μg/ml Leupeptin, 5 μg/ml Pepstatin A, 5 μg/ml Benzamidine, 12 μg/ml AEBSF). Cells were broken up with an ice cold Dounce homogenizer. After mild centrifugation (5 min, 500 g) to remove unbroken cells and the nuclei the supernatant was ultracentrifuged at 100000 g for 45 minutes at 4°C. The pellet representing the membrane fraction and the supernatant representing the cytosolic fraction were resuspended in protein sample buffer.

Immunoprecipitation

Neutrophils were lysed in ice cold lysis buffer (20 mM Tris-HCl pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 0.5% NP40, 10% (v/v) glycerol, 0.5 mg/ml BSA, 1 mM Na₃VO₄, 1 mM PMSF, 21 μg/ml aprotinin, 12 μg/ml AEBSF, 5 μg/ml leupeptin, 5 μg/ml pepstatin A, and 5 μg/ml benzamidine). A cleared cell lysate was received by rapid centrifugation for 15 min at 4 °C. For immunoprecipitation, the lysates was preincubated with

the appropriate antibodies for 2 h, before protein A sepharose beads were added for another 2 h. Finally, the beads were washed three times in ice-cold lysis buffer.

Immunoblotting

Equal amounts of proteins were separated by SDS-PAGE via 10% polyacrylamide gels and subsequently transferred to PVDF (polyvinylidene fluoride) membranes. Blots were incubated in NET-G buffer (Tris-HCl pH 8.0, 10 mM NaCl, 150 mM EDTA, 5 mM Tween-20, 0.05% (v/v) ; and 0.05 % (g/v) gelatine for 1 h. Immunoblotting was performed using the indicated primary antibodies in NET-G buffer. After subsequent incubation with horseradish peroxidase-conjugated secondary antibody, the blots were quantitatively assayed using Western Lightning chemiluminescence detection (Perkin-Elmer Life Sciences, Boston, USA) and a LAS3000 CCD camera (Fujifilm, Düsseldorf, Germany). Densitometric analysis of specific bands was used for the statistical analysis.

ROS production in neutrophils

Production of reactive oxygen species was monitored using lucigenin-dependent chemiluminescence. Neutrophils were resuspended to a density of 1×10^6 cells/ml, containing 200 μ M lucigenin. Measurements were performed in triplicate at 37 °C [14].

RESULTS

PI3K γ associates with PKC α

Using a monoclonal antibody specific to the p110 catalytic subunit of PI3K γ [8], we initially produced immunoprecipitates from different cell types. Analyses of these precipitates by mass spectroscopy revealed numerous p110 γ cell-specific interaction partners. The PI3K γ immunoprecipitates obtained from human neutrophils were separated via SDS-PAGE. Copurified proteins were subsequently trypsin digested and identified via mass spectrometry. In the 75 kDa molecular weight range, we identified peptides that originated from PKC α . In addition, we found indication for association of matrix metalloproteinase MMP 9, HSP70, and alpha subunit of heterotrimeric Gi proteins to the immunoprecipitated complex. To validate the interaction of PI3K γ with PKC α , we analyzed immunoprecipitates of PI3K γ by western blotting for the presence of PKC α (Fig. 1A). These data revealed that PKC α was a specific interaction partner of PI3K γ in human neutrophils.

In order to validate the interaction of PI3K γ and PKC α *in vitro*, identical amounts of His epitope-labeled recombinant PI3K γ were incubated with GST epitope-labeled PKC α . Purification of the tagged proteins was carried out using Ni-NTA agarose beads for His-PI3K γ and glutathione sepharose beads for GST-PKC α . As a control for nonspecific binding to the column material, identical reaction mixtures were prepared excluding the corresponding bait proteins. While in the His-PI3K γ pull-down experiment PKC α was efficiently copurified, in the absence of His-PI3K γ , only traces of GST-PKC α bound to the Ni-NTA agarose column. On the other hand, glutathione beads copurified His-PI3K γ via GST-PKC α . Only traces of His-PI3K γ could be detected if PKC α was omitted (Fig. 1B), or if the GST epitope alone was used as a bait protein (data not shown). In another experiment, His-PI3K γ and GST-PKC α were coexpressed in Sf9 insect cells. After 72 h, the cells were lysed and the cleared lysates were applied to GST- or His-specific pull-down assays. Essentially, the interaction profiles obtained were identical to those resulting from the biochemical studies described above

(Suppl. Fig. 1). These results indicate that the association of PI3K γ with PKC α is direct and specific.

PI3K γ phosphorylates and stimulates PKC α *in vitro*

The direct binding of PI3K γ to PKC α suggests that trans-phosphorylation reactions may occur between these signalling proteins. Both PKC α and PI3K γ have been shown to exhibit protein kinase activities. Incubation of the recombinant proteins in the presence of [γ - 32 P]-ATP did not reveal PKC α -mediated phosphorylation of PI3K γ (data not shown). In contrast, pronounced phosphorylation of PKC α by PI3K γ could be demonstrated (Fig. 2A). To exclude auto-phosphorylation of the enzyme, PKC α activity was suppressed using BIM as an inhibitor of classical PKC species. Unaltered incorporation of 32 P confirmed PI3K γ -mediated phosphorylation of the protein. Impaired phosphorylation of PKC α in the presence of the enzymatically inactive variant PI3K γ -KR or the PI3K γ inhibitor wortmannin verified PI3K γ -mediated trans-phosphorylation of PKC α (Fig. 2A).

Activation of PKC α by other protein kinases, such as PDK1, has been described as involving the successive phosphorylation of amino acid residues Thr497, Thr638, and Ser657 in the kinase domain [16,17]. These target sites were analyzed after PI3K γ -mediated phosphorylation. Both recombinant proteins were incubated in the presence of nonradioactive ATP, and PKC α was repurified using SDS-PAGE, extracted from the PAA gel, trypsinized, and subjected to mass spectrometry. While the kinase reaction in the absence of PI3K γ or ATP did not result in phosphorylated amino acid residues, coincubation with PI3K γ revealed peptides with specific mass shifts, suggesting the specific trans-phosphorylation of PKC α . Phosphorylation of Ser or Thr could be identified at 3 different regions of the protein (Suppl. Fig. 2). The most pronounced phosphorylation (residues Thr48, Ser51, and/or Thr54) according to number of identified peptides and the score of these peptides was identified in peptide QPTFCSHCTDFIWGF GK (amino acid residues 46–62),

which is localized in the second C1 domain of the DAG-binding domain of PKC α . Two less abundant phosphorylation clusters have been found in the catalytic domain of the protein (Suppl. Fig. 2). These data confirm the serine–threonine kinase activities of PI3K γ , and explore, for the first time to our knowledge, a specific site that is trans-phosphorylated by the enzyme.

The putative PI3K γ -specific phosphorylation site of PKC α has some similarities to the auto-phosphorylation site of PI3K γ , which was recently identified by Czupalla et al. [18]. As shown in Fig. 2B there is distinct homology between amino acids 36–61 of PKC α and the auto-phosphorylation site located at the C terminus of PI3K γ . The resemblance between these two target sites for PI3K γ protein kinase activity prompted us to search for further homology patterns in proteins that have been shown to act as substrates for PI3K γ protein kinase. In recent reports, PI3K γ -specific phosphorylations of MEK [19–21], p101 [21], Ras [22], 4EBP1 [22], and tropomyosin [23] were detected using recombinant proteins and cellular systems. Indeed, all of these PI3K γ protein kinase substrates exhibit peptide sequences with different degrees of similarity to the PI3K γ auto-phosphorylation site [18] and the trans-phosphorylation site in PKC α that was identified in the present report (Fig. 2B).

After elucidating the binding and trans-phosphorylation of PKC α by PI3K γ , we investigated the effects of the interaction of both signalling proteins on the specific activity of PKC α in vitro. The assay was carried out using histone H1 as a substrate, and PKC α kinase activity was monitored by incorporation of [γ - 32 P]-ATP–derived phosphate. While PI3K γ alone did not phosphorylate histone H1, 32 P was efficiently incorporated in the presence of PKC α . This reaction could be specifically inhibited by addition of the PKC inhibitor BIM (Fig. 2C). We next explored the effects of PI3K γ on PKC α activity. As shown in Fig. 2D, a pronounced stimulation of substrate phosphorylation was observed in the presence of PI3K γ in a time range up to 20 min.

We further explored the NADPH oxidase components p47phox and p67phox as substrates in PKC α kinase assays. While p47phox was phosphorylated with similar efficiency to histone H1 (Fig. 2E), phosphorylation of p67phox was only marginal (data not shown). Preincubation of PKC α with PI3K γ induced only weak stimulation of p47phox phosphorylation compared with the kinase reaction in the absence of PI3K γ .

fMLP induces PI3K γ and PKC α complex formation in human neutrophils

To gain insight into the physiological role of the interactions between PI3K γ and PKC α , we studied the complex formation of these proteins following stimulation of neutrophils with fMLP. PI3K γ was immunoprecipitated at the indicated time points, and coprecipitated PKC α was assayed by western blotting (Fig. 3). The addition of fMLP markedly increased PKC α association with PI3K γ in a transient manner (Fig. 3A). In unstimulated neutrophils we did not observe any alteration in PKC α binding to the immunoprecipitate in the given period (Fig. 3B). Remarkably, the p47phox subunit of NADH oxidase associated with identical kinetics to this complex (Fig. 3A).

It has been demonstrated in different cellular systems that activation of both PI3K γ and PKC α causes the association of these proteins with the plasma membrane [24, 25]. To correlate this process with the fMLP-induced interaction of the signalling proteins, we analyzed the kinetics of membrane association after fMLP stimulation. The data shown in Fig. 3C demonstrate that the distribution of both proteins was altered in response to fMLP stimulation. While PI3K γ segregated to the membrane fraction, peaking after 120 s of stimulation, most PKC α molecules associated with membranes 60 s after fMLP treatment. These data reveal that the formation of fMLP-mediated PI3K γ -PKC α complexes is a process paralleling the membrane association of these proteins.

fMLP induces PI3K γ -mediated activation of PKC α

We next explored the functional relevance of the fMLP-induced association of PI3K γ , PKC α , and p47phox. Classical PKC species have been shown to stimulate NADH oxidase activity via phosphorylation of p47phox [26]. However, the signalling reactions affecting PKC activity in neutrophils are poorly understood. Here, we first analyzed specific PKC α activity in response to fMLP stimulation. Neutrophils were stimulated for the indicated time points, aliquots were withdrawn and lysed, and PKC α was immunoprecipitated. The protein kinase activity of PKC α was analyzed by protein kinase assay using histone H1 as a substrate for ^{32}P incorporation. As shown in Fig. 4A, specific PKC α kinase activity was markedly enhanced in response to neutrophil stimulation by fMLP. Maximum activity was reached 30 sec after fMLP addition, which correlates with the onset of PI3K γ /PKC α /p47phox complex formation.

To test the involvement of PI3K γ enzymatic activity in the fMLP-dependent stimulation of PKC α , PKC α activity was analyzed in cells treated with the specific PI3K γ inhibitor AS605240 [27]. While the untreated cells corroborated the activity profile shown in Fig 4A, PKC α immunoprecipitated from neutrophils after treatment with AS605240 exhibited notably impaired PKC activity (Fig. 4B). This observation points to a strict dependence of PKC α activity on functionally active PI3K γ . To exclude nonspecific effects of AS605240 on PKC α kinase activity, recombinant PKC α was preincubated with PI3K γ inhibitors and subsequently assayed for its protein kinase activity *in vitro*. While treatment with the PKC inhibitor BIM completely abolished PKC α activity, neither wortmannin nor AS605240 significantly affected the enzymatic activity of PKC α (Fig. 4C).

Enzymatic activities of PI3K γ and PKC α control fMLP-dependent ROS production in neutrophils

A role for PI3K γ in the production of ROS was elucidated in detail using specific inhibitors and PI3K γ -deficient mice [28]. To study the involvement of PKC α in the control of oxidative

bursts induced by fMLP, human neutrophils were treated with BIM, and the kinetics of ROS production were recorded. While the untreated cells showed the expected ROS induction profile after fMLP treatment, ROS production in BIM-pretreated cells was severely diminished (Fig. 5). PKC inhibitor Gö6976 applied at the same concentration showed essentially similar abrogation of ROS production (data not shown). PI3K γ inhibition by AS605240 reduced fMLP-mediated ROS production to a degree similar to BIM. These data demonstrate the involvement of PKC α and PI3K γ enzymatic activities in fMLP-mediated ROS induction.

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DISCUSSION

There is an increasing amount of evidence indicating that the differential interactions of PI3K γ with other signalling proteins depend upon the cell type [2, 25, 29]. Building upon these initial findings, we searched for PI3K γ binding partners in different human cell types. Mass spectroscopy of PI3K γ immunoprecipitates obtained from human neutrophils revealed PKC α as a novel interaction partner of PI3K γ . Analysis of PI3K γ binding to PKC α *in vitro* clearly indicated the direct and specific association of these two signalling proteins. In addition, we demonstrated PKC α phosphorylation by PI3K γ protein kinase activity. These results expand the list of substrates known to be phosphorylated by PI3K γ protein kinase activity under *in vitro* conditions. In addition to substantial auto-phosphorylation activity, the current list includes trans-phosphorylation of MEK, p101, H-Ras, and tropomyosin [10;19-23]. In spite of preliminary data hinting at a regulatory function for the PI3K γ protein kinase in cellular systems [20], compelling evidence of a physiological role for this enzymatic activity is still missing.

Serine and threonine residues phosphorylated by PI3K γ have been identified in three regions of PKC α . Interestingly, the most pronounced phosphorylation was found in a PKC α peptide localized the second C1 domain of the DAG-binding domain of the protein. It shows significant homology to the C-terminal end of PI3K γ , which has been identified as the auto-phosphorylation site [18]. Further similarities of this putative trans-phosphorylation site in PKC α have been identified to potential phosphorylation sites in other proteins in MEK, p101, Ras, 4EBP1, and tropomyosin. All of these intracellular proteins have been found to act as substrates for PI3K γ protein kinase activity [19-23]. The homology of the PKC α phosphorylation site to other known substrates suggests a widespread functional relevance of trans-phosphorylation reactions catalyzed by PI3K γ protein kinase activity.

In our hands, the *in vitro* interactions of PKC α with PI3K γ induced a marked increase in PKC α activity. Part of the stimulatory effect was observed after addition of a kinase-negative mutant of PI3K γ , supporting the hypothesis that association with PI3K γ directly augments

PKC α activity. Together with our *in vitro* investigations, these data provide evidence of the direct and specific binding of PI3K γ to PKC α , and identify PKC α as a potential substrate, whose enzymatic activity can be stimulated by phosphorylation triggered by PI3K γ protein kinase activity. It can not be excluded that immunoprecipitated PKC α contained additional proteins affecting total kinase activity, but since ^{32}P incorporation in the PKC α substrate histone H1 was drastically abrogated in presence of PI3K γ selective inhibitor AS605240, a specific PI3K γ mediated stimulation of PKC α activity can be concluded.

To explore the physiological function of PI3K γ and PKC α interactions in human neutrophils, we stimulated the cells with the bacterial peptide fMLP. fMLP has been shown to affect both directed neutrophil migration and ROS production. Comparative investigations of neutrophils from PI3K γ -deficient mice have demonstrated that both fMLP-dependent processes are mediated by PI3K γ [4–6]. PKC α has been described as a mediator of fMLP-induced oxidative burst, and the regulatory subunit of NADH oxidase p47phox was identified as an important target of PKC α activity [26]. The signalling pathway linking fMLP, the fMLP receptor, and PKC α is poorly understood. Thus, our experimental data regarding the fMLP-dependent association of PKC α with PI3K γ provide a first clue for signalling reactions that induce stimulation of PKC α activity by fMLP.

The fMLP-induced interaction between PI3K γ and PKC α and the translocation of both proteins to the plasma membrane may contribute to the stimulation of PKC α activity observed after agonist addition. The PKC α substrate p47phox joins the complex with similar kinetics, possibly facilitating phosphorylation of this subunit and subsequent stimulation of NADH oxidase. As shown by our inhibition experiments, PI3K γ enzymatic activity is an essential mediator of fMLP-dependent stimulation of PKC α activity in neutrophils. Due to the lack of specific inhibitors, these results do not allow for a definitive correlation of the blocking effect of AS605240 on PI3K γ lipid or protein kinase activities. However, our findings showing PKC α phosphorylation by PI3K γ protein kinase *in vitro*, and the concomitant increase of PKC α activity, provide evidence for a lipid kinase-independent signalling reaction.

This conclusion may be in accordance with recent findings by Suire et al. [7]. These authors initially investigated the fMLP-dependent synthesis of PIP₃ (the product of PI3K γ lipid kinase activity) in p101-deficient mouse neutrophils. Compared with wild-type neutrophils, they observed a massive decrease in fMLP-sensitive PIP₃ synthesis, indicating an essential role for the p101 adapter protein in the control of PI3K γ lipid kinase activity by G protein-coupled receptors. As a second parameter, Suire et al. also performed comparative analysis of fMLP-dependent oxidative burst in wild-type and p101-deficient neutrophils [7]. Surprisingly, they did not observe any significant differences in ROS production indicating a marginal role for p101 in fMLP/PI3K γ -dependent ROS production. Our investigations suggest the involvement of PI3K γ protein kinase activity in the control of NADH oxidase activity. They also provide evidence for a consecutive signalling path: fMLP→fMLP receptor→PI3K γ protein kinase→PKC α →p47phox→NADH-oxidase, which does not involve p101/p110 γ -dependent PIP₃ production.

In fMLP-induced signalling pathways, PI3K γ may act as a bifurcation point. Whereas PIP₃ produced by PI3K γ lipid kinase activity controls the migration induced by chemoattractants, PI3K γ protein kinase activity may affect the oxidative burst via phosphorylation and concomitant stimulation of PKC α . Hence, these findings provide additional evidence for the differential cell-specific regulatory functions of PI3K γ .

ACKNOWLEDGEMENTS

This work was supported by the Deutsche Forschungsgemeinschaft SFB 604. KL and JPM contributed equally to this work.

FIGURES

FIGURE 1: PI3K γ binds PKC α

(A) PI3K γ and PKC α associate in human neutrophils. Freshly isolated human neutrophils were aliquoted ($3\text{--}4 \times 10^7$ cells) and treated with $1 \mu\text{M}$ fMLP. A whole-cell lysate was prepared and equal amounts of protein were immunoprecipitated with PI3K γ antibody and immunoblotted with an antibody for PKC α . Antibodies against the above-mentioned proteins were precipitated with Protein A agarose beads, extensively washed, subjected to SDS-PAGE, and blotted. Bait and cobound proteins were analyzed immunologically using specific antibodies (lane 1). To show nonspecific binding, primary antibodies were replaced by nonspecific IgG in a parallel sample (lane 2).

(B) PI3K γ and PKC α associate in vitro. Purified recombinant His-PI3K γ and GST-PKC α protein ($1 \mu\text{g}$ each) were incubated in $50 \mu\text{l}$ of protein kinase buffer for 30 min at room temperature. Complex formation was analyzed using $50 \mu\text{l}$ of glutathione beads to purify GST-PKC α (PKC α pull down) or $50 \mu\text{l}$ of Ni-NTA beads to purify His-PI3K γ (PI3K γ pull down). After further incubation for 30 min, extensively washed beads were subjected to SDS-PAGE and blotted. Bait and cobound proteins were analyzed immunologically using specific antibodies. To show nonspecific binding, bait proteins were omitted from a parallel sample as indicated. Representative data are shown of experiments that were repeated several times.

FIGURE 2: PI3K γ phosphorylates PKC α and stimulates its activity

(A) Phosphorylation of PKC α by PI3K γ . His-PI3K γ (WT, wild type; KR, kinase inactive) and GST-PKC α protein ($1 \mu\text{g}$ each) were incubated in $50 \mu\text{l}$ of protein kinase buffer for 15 min at 37°C in the presence of $2 \mu\text{Ci}$ of $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$. As a control, the PKC inhibitor BIM (100 nM) or the PI3K γ inhibitor wortmannin (WM, 100 nM) were added to the reaction mixture as indicated. Proteins were separated by SDS-PAGE. ^{32}P incorporated into GST-PKC α was detected and quantified by autoradiography using a phospho-imaging system.

Representative data of experiments repeated three times are shown. Data are means \pm standard deviations (S.D.). Statistical significance was calculated using unpaired T-Test, * $P < 0.05$, ** $P < 0.01$.

(B) Alignment of putative phosphorylation sites of PI3K γ protein kinase activity.

(C) Histone H1 is a specific substrate for PKC α . GST-PKC α or His-PI3K γ (1 μ g each) were incubated in 50 μ l of protein kinase buffer containing the PKC α substrate histone H1 for 15 min at 37 $^{\circ}$ C in the presence of 2 μ Ci of [γ - 32 P]-ATP. Phosphorylation of histone H1 was monitored by separation via SDS-PAGE. 32 P incorporation into histone H1 was quantified by autoradiography using a phospho-imaging system. As controls, the PKC inhibitor BIM (100 nM) was added, or PI3K γ was excluded from the reactions. The results are representative of three experiments. Quantification data are means \pm S.D.

(D) Specific activity of PKC α increases in presence of PI3K γ . GST-PKC α protein and His-PI3K γ were incubated for 30 min at 37 $^{\circ}$ C in the presence of 30 μ M ATP. Subsequently, the reaction mixture was diluted 6-fold, histone H1 and [γ - 32 P]-ATP were added, and samples were withdrawn at the indicated time points. Phosphorylation of histone H1 was analyzed as described above. Representative data are shown of experiments that were repeated several times. Quantification data are means \pm S.D.

(E) p47phox is a substrate of PKC α . Purified p47phox, GST-PKC α , and His-PI3K γ proteins were incubated at 37 $^{\circ}$ C in the presence of 30 μ M ATP. Subsequently, the reaction mixture was diluted 6-fold, histone H1 and [γ - 32 P]-ATP were added, and samples were withdrawn at the indicated time points. Phosphorylation of p47phox was analyzed as described above. Representative data are shown of experiments that were repeated several times. Quantification data are means \pm S.D.

FIGURE 3: fMLP-dependent interaction of PI3K γ and PKC α in human neutrophils

(A, B) fMLP-induced interaction of PI3K γ with PKC α . Freshly isolated human neutrophils were aliquoted ($3\text{--}4 \times 10^7$ cells) and treated with $1 \mu\text{M}$ fMLP (A) or left unstimulated (B) for the indicated times. Whole-cell lysates were prepared and equal amounts of protein were immunoprecipitated with PI3K γ antibody and immunoblotted with an antibody for PKC α . The results are representative of three experiments.

(C) fMLP-induced translocation of PI3K γ and PKC α to the plasma membrane. Freshly isolated human neutrophils were aliquoted ($3\text{--}4 \times 10^7$ cells) and treated with $1 \mu\text{M}$ fMLP for the indicated times. After incubation, cells were processed for membrane and cytosol preparation as described in the text. Membrane and cytosolic fractions were immunoblotted with PI3K γ and PKC α antibody. The results are representative of three experiments.

FIGURE 4: Stimulation of human neutrophils induces PI3K γ -mediated activation of PKC α

(A) fMLP-induced PKC α activity. Freshly isolated human neutrophils ($3\text{--}4 \times 10^7$ cells) were treated with $1 \mu\text{M}$ fMLP. Samples were withdrawn at the indicated time points, lysed, and subjected to immunoprecipitation of PKC α . PKC α kinase activity was determined with SDS-PAGE and autoradiography using a phospho-imaging system to analyze ^{32}P incorporation into histone H1. Specific kinase activity was quantified as the ratio of ^{32}P incorporation to immunoprecipitated PKC α , as indicated in the graph. Results are means \pm S.D. * Result significantly different to untreated cells ($P < 0.05$).

(B) Effects of PI3K γ inhibitor on fMLP-dependent PKC α activity. Neutrophils were preincubated for 30 min at 37°C in the presence or absence of the PI3K γ -specific inhibitor

AS605240 (10 μ M) before stimulation with 1 μ M fMLP. PKC α immunoprecipitation and quantification of specific PKC α kinase activity were performed as described above.

(C) AS605240 does not inhibit PKC α activity. In vitro purified GST-PKC α (1 μ g) was incubated in 50 μ l of protein kinase buffer for 15 min at 37 °C in the presence of 2 μ Ci of [γ - 32 P]-ATP in the presence of the PKC inhibitor BIM (100 nM), the PI3K γ inhibitor wortmannin (100 nM), or AS605240 at the indicated concentrations. Proteins were separated by SDS-PAGE. 32 P incorporated into histone H1 was detected and quantified by autoradiography using a phospho-imaging system. The data presented are representative of three experiments.

FIGURE 5: PI3K γ - and PKC α -dependent ROS production in human neutrophils

Freshly isolated human neutrophils were isolated and diluted to 1×10^6 cells in Hanks buffered solution/0.1% BSA. Cells were incubated for 30 min at 37 °C in the absence or presence of inhibitors (AS605240, 1 μ M or BIM, 1 μ M). Lucigenin-enhanced chemiluminescence was measured after stimulation with 1 μ M fMLP. Measurements were done in triplicate. The data presented are representative of three experiments.

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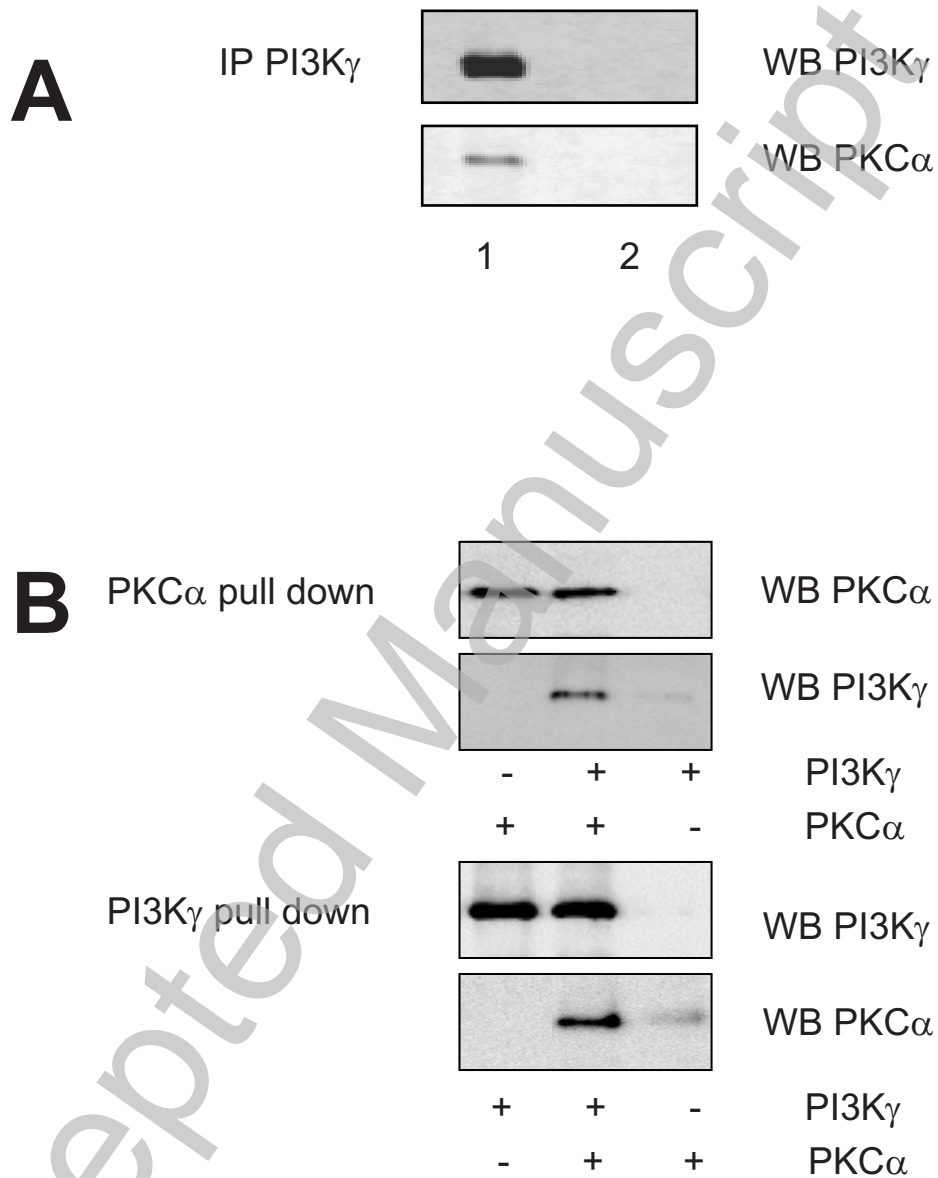


Figure 1

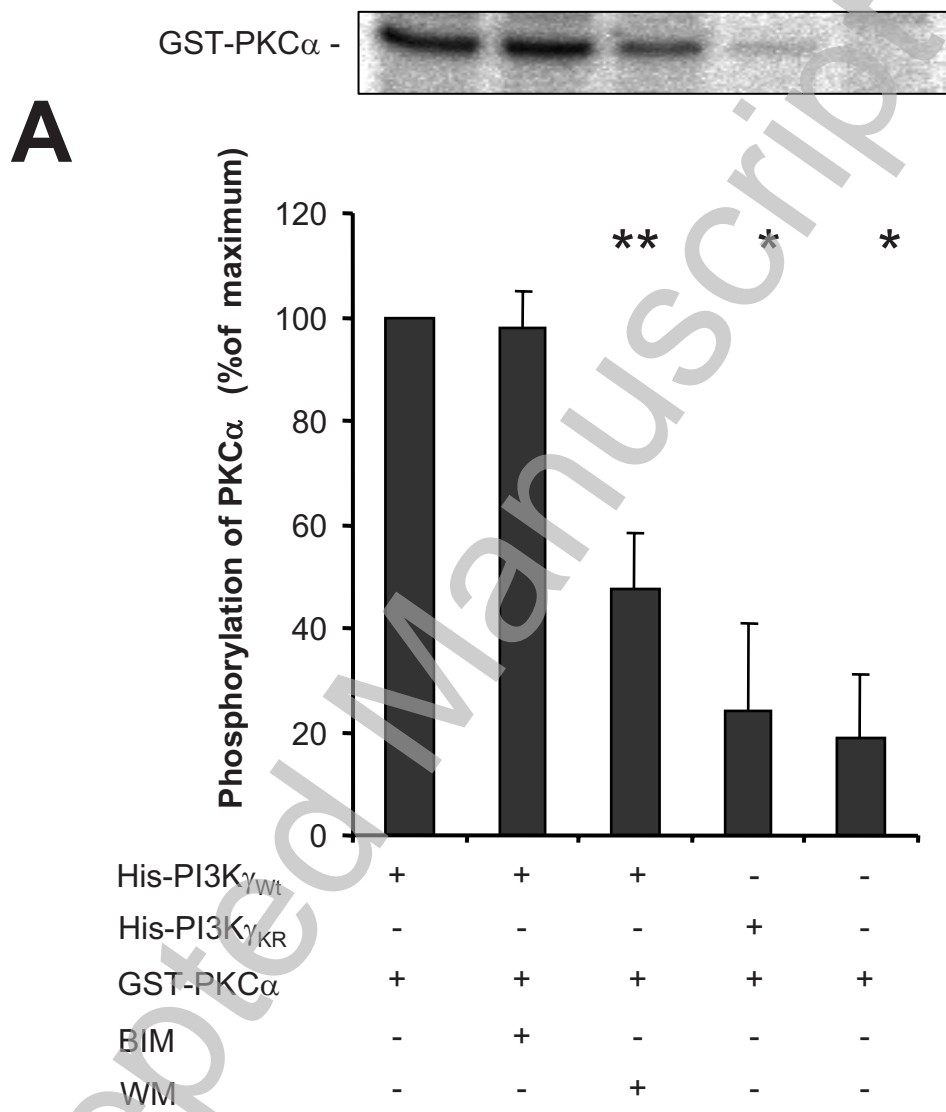


Figure 2

B

PI3K γ	(aa 1088)	LHLVLGI KQ GEK HS A
PKC α	(aa 38)	KFIARFF KQ PTFC SH CTDFIW
MEK-1	(aa 228)	SYMSPERL Q GTHY SV QSDIWS
p101	(aa 755)	NLNKACR KQ EEL DS SMEALTL
tropomyosin	(aa 202)	NNLKSLEA Q AEKY SQ KEDRYE
H-Ras	(aa 123)	RTVESRQA Q DLAR SY GIPYIE
4EBP1	(aa 99)	RNSPEDKRAGGEE SQ FEMDI

Figure 2

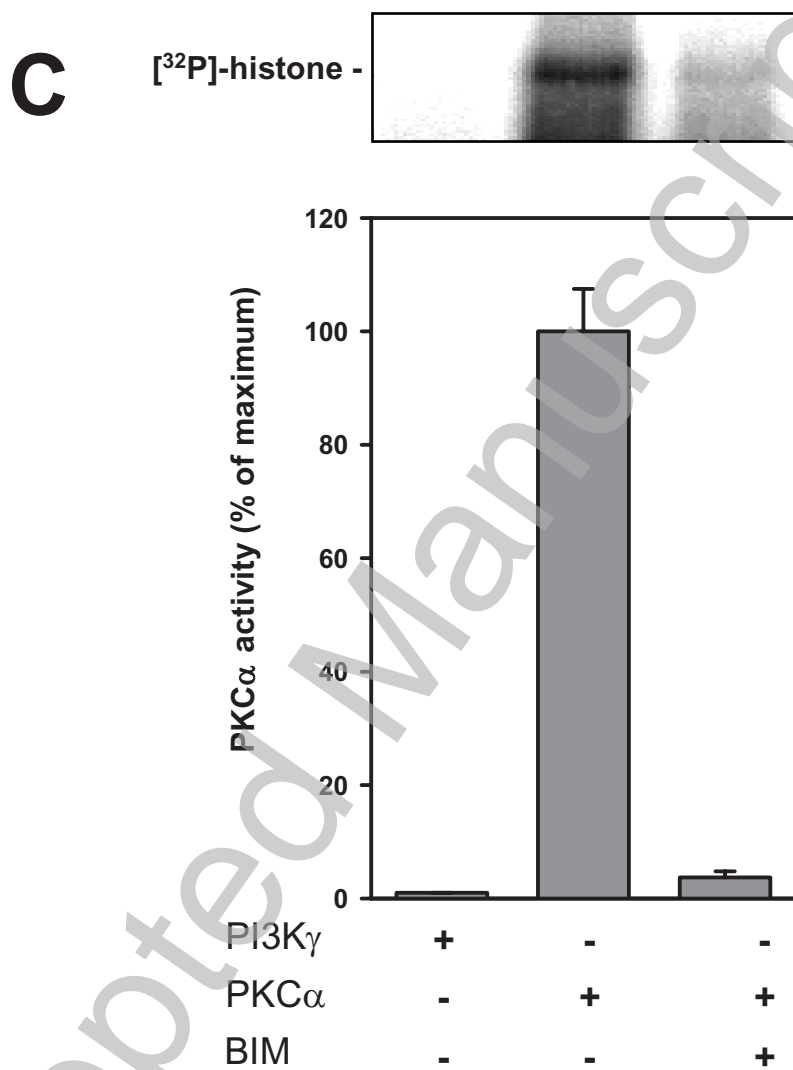


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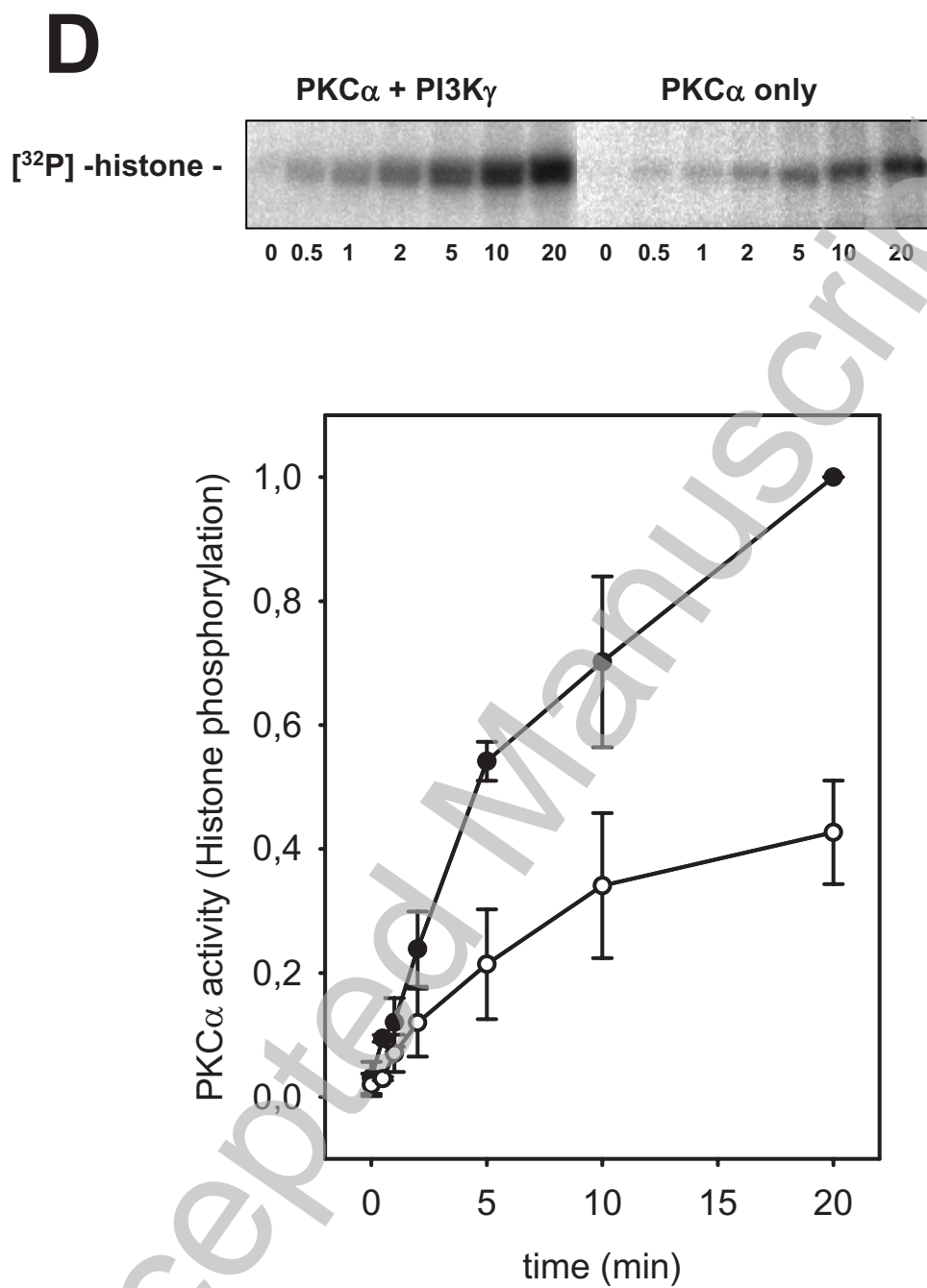


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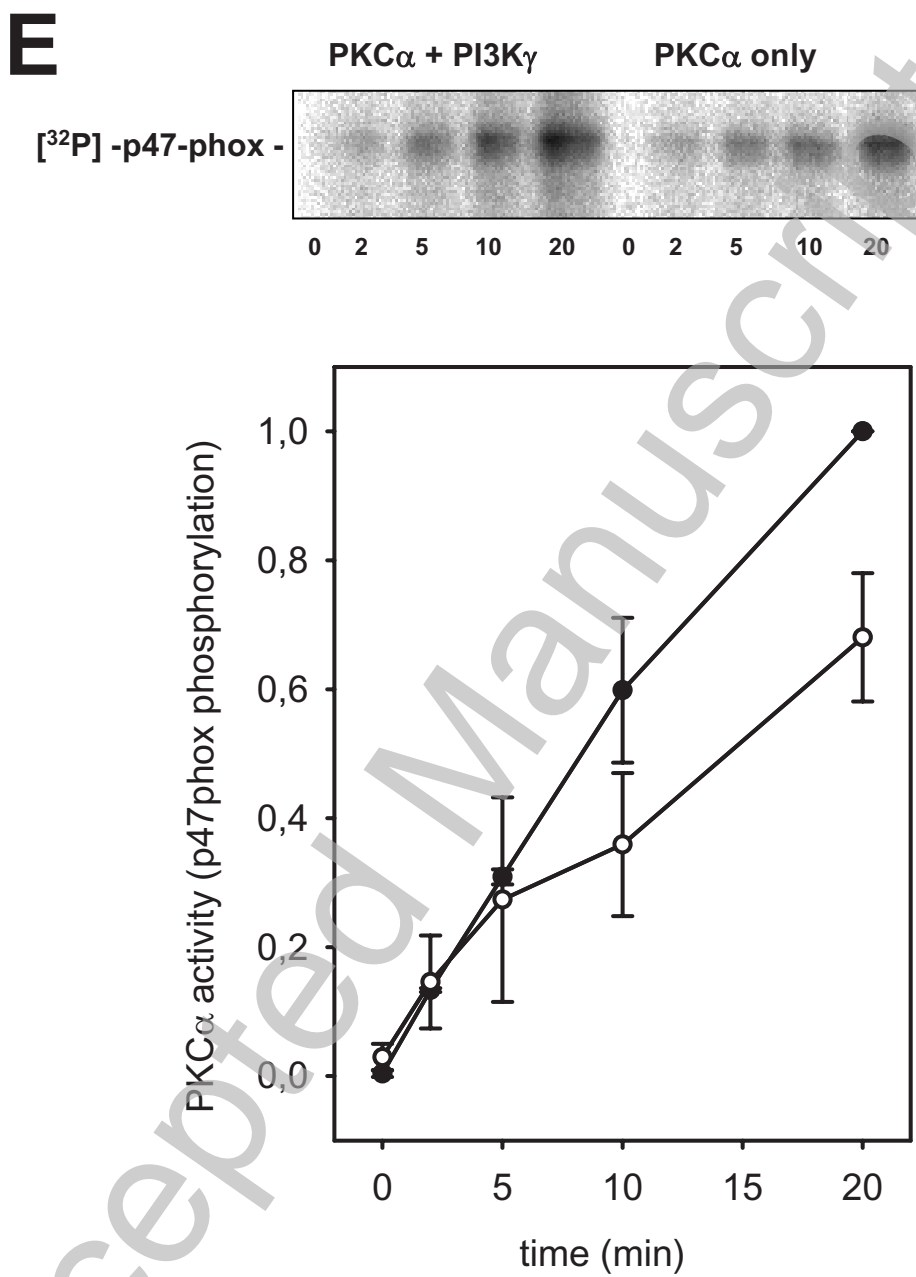


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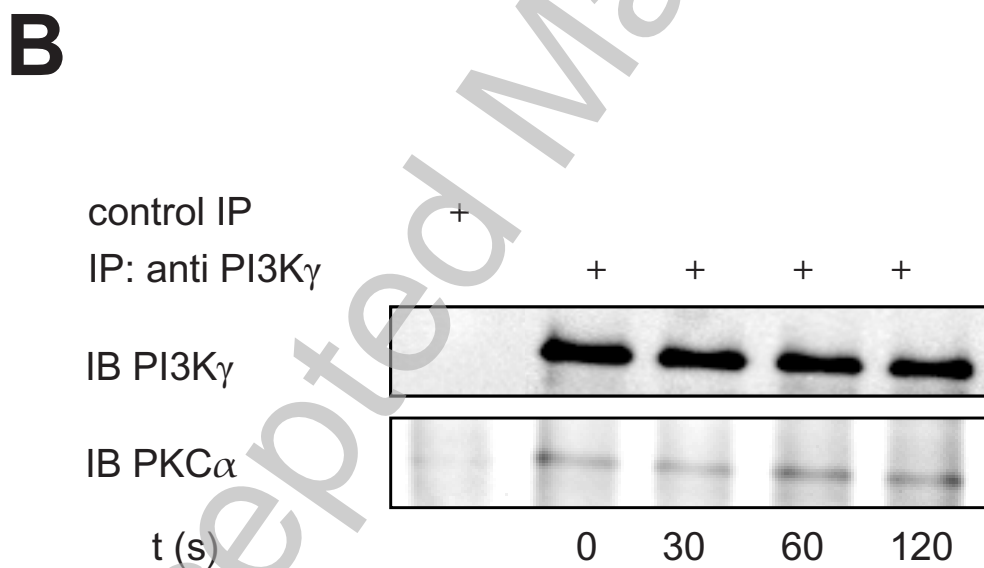
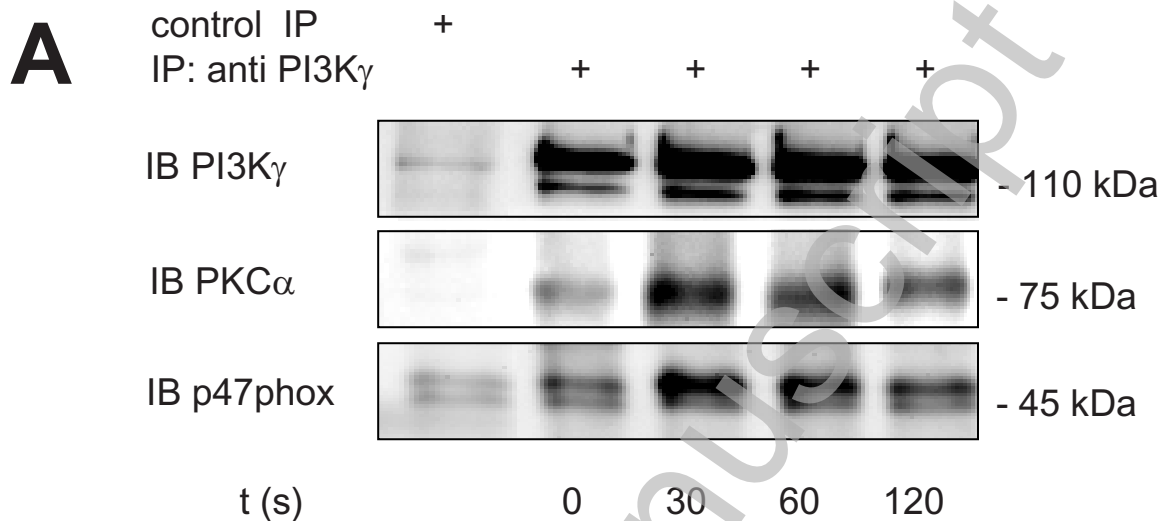


Figure 3

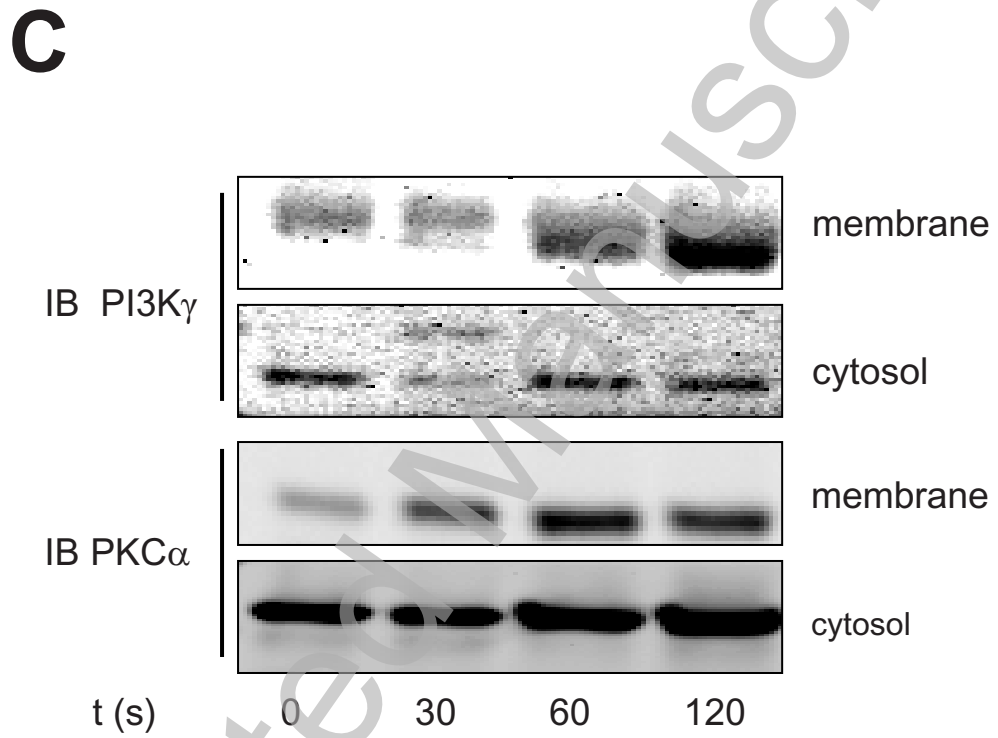


Figure 3

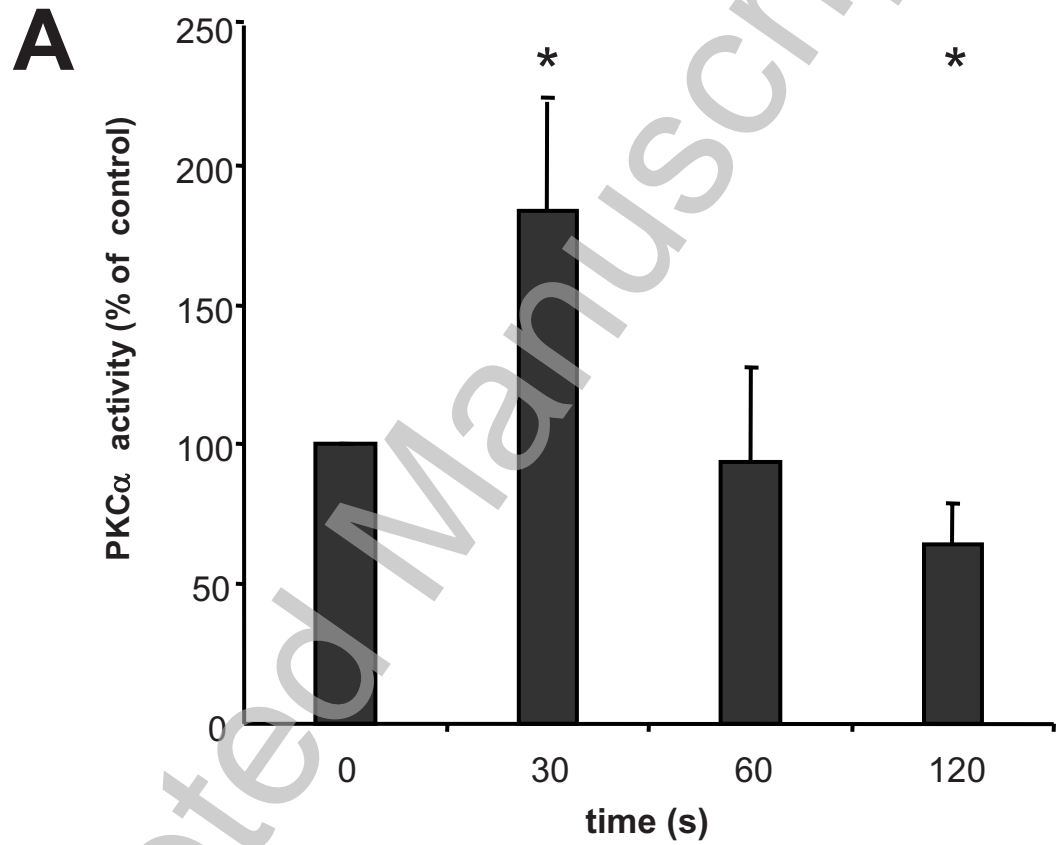


Figure 4

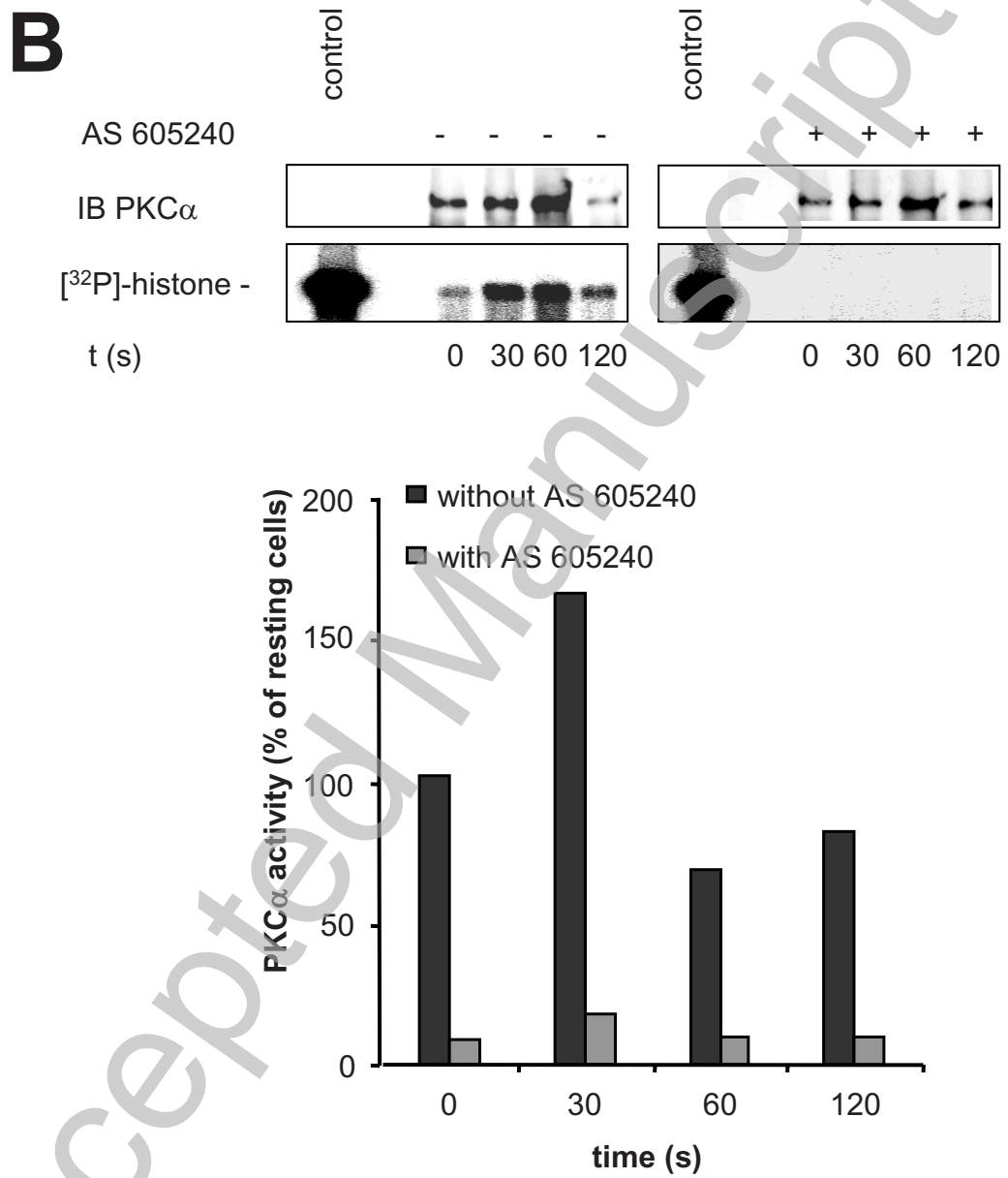


Fig. 4

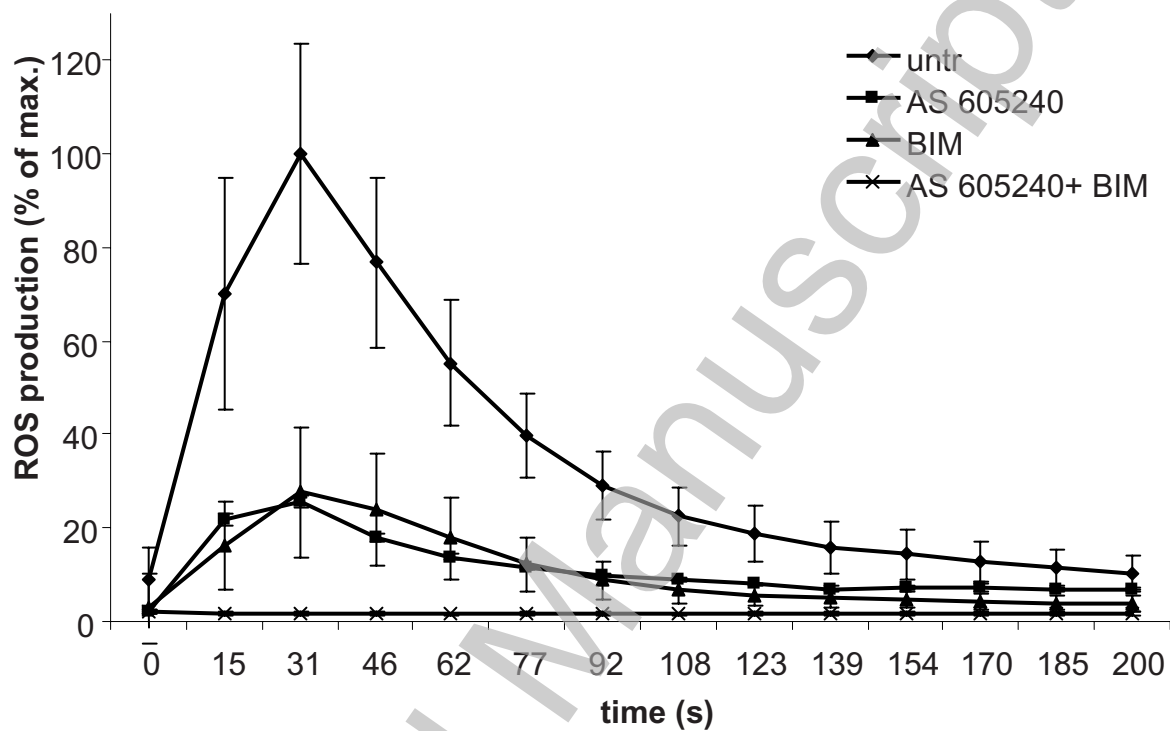


Figure 5