RESEARCH COMMUNICATION

Co-operation of phosphatidylinositol transfer protein with phosphoinositide 3-kinase γ in the formylmethionyl-leucylphenylalanine-dependent production of phosphatidylinositol 3,4,5-trisphosphate in human neutrophils

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Phosphoinositide 3-kinase (PI3K) and its product phosphatidylinositol 3,4,5-trisphosphate (PIP_3) play an essential role in the regulation of neutrophil functions by the chemoattractant formylmethionyl-leucylphenylalanine (FMLP). Here we show that permeabilization of human neutrophils leads to loss of cytosolic components, including PI3Kγ, and causes the loss of FMLP-dependent production of PIP_3. FMLP-sensitive synthesis of PIP_3 could be restored by reconstitution of permeabilized neutrophils with recombinant PI3Kγ. Admixture of recombinant phosphatidylinositol transfer protein (PITP) to the reconstitution cocktail produced a further increase of PIP_3 synthesis, whereas pertussis toxin suppressed the FMLP-dependent production of PIP_3. We conclude that FMLP-sensitive PIP_3 formation in human neutrophils involves the FMLP receptor, heterotrimeric G-proteins of the G_i type, PI3Kγ and PITP.

INTRODUCTION

Human neutrophils phagocytose and destroy invading bacteria. In addition, they undergo shape change, chemotaxis, degranulation and the production of superoxide anions when stimulated by bacterially derived formylated peptides such as formylmethionyl-leucylphenylalanine (FMLP). FMLP-induced degranulation and production of superoxide is suppressed by wortmannin, an inhibitor of phosphoinositide 3-kinase (PI3K) [1–3]. This finding, and recent studies of the FMLP-dependent phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP_2) to phosphatidylinositol 3,4,5-trisphosphate (PIP_3) [4,5] suggest the involvement of a G-protein-dependent PI3K and its product, PIP_3, in the signalling cascade stimulated by FMLP in neutrophils. A novel form of PI3K, termed PI3Kγ, which can be activated *in vitro* by α and $\beta\gamma$ subunits of G-proteins, has been cloned recently [6].

The *in vivo* substrate for PI3K, PIP_2, is synthesized from phosphatidylinositol (PI) via sequential phosphorylation by PI 4-kinase and PI 4-phosphate (PI4P) 5-kinase, and phosphatidylinositol transfer protein (PITP) has been identified as a cofactor in this process [7,8]. Additionally, PITP is required for phospholipase Cβ- and Cγ-dependent signalling pathways [9,10,7], exocytosis [11–13] and vesicle formation [14]. As a molecular basis of the multifold effects of PITP, two functions have been proposed. First PITP is able to transfer PI or phosphatidylcholine from one cellular compartment to another, causing an equilibrium of both lipids in intracellular membranes [15]. In this way PITP is able to induce a net transfer from PI-producing to PI-consuming compartments like the plasma membrane. Furthermore, an important function of PITP as a cofactor of several enzymes of the PI metabolism has been suggested [13,16].

To explore the requirement of both PI3Kγ and PITP in FMLP-stimulated human neutrophils, we have investigated their ability to reconstitute receptor-dependent PIP_3 production in permeabilized cells. Our results demonstrate that, in this reconstitution system, PI3Kγ can be stimulated by additon of FMLP. FMLP-dependent PIP_3 production can be suppressed by pertussis toxin and is considerably enhanced in the presence of PITP. The results point to a signalling pathway from FMLP, via the FMLP receptor, G_i species of heterotrimeric G-proteins to PI3Kγ. PITP is required for PIP_3 synthesis catalysed by PI3Kγ.

MATERIALS AND METHODS

Permeabilization of neutrophils

Human neutrophils were freshly isolated as described in [5]. Extracellular nucleotide-sensitive receptors were inactivated by mixing a suspension of the cells [$(2–4) \times 10^7$ cells/ml] in Pipes/saline buffer (20 mM Pipes/137 mM NaCl/3 mM KCl, pH 7.4) with 0.15 vol. of freshly prepared 0.3 M dithiothreitol and 3.85 mM ATP [5]. After 5 min end-on-end mixing at room temperature, cells were diluted 5-fold and then washed three times with Pipes/saline buffer. The neutrophils were resuspended in Pipes/saline and challenged with permeabilization cocktail, composed of 0.6 unit/ml streptolysin O, 3 mM $MgCl_2$, 0.3 mM $CaCl_2$ and 1 mM EGTA (final concns.) in a total volume of 500 μl [5]. This solution possesses a free ionized calcium concentration of 100 nM. Cells were incubated for 40 min at 37 °C, washed once in ice-cold Pipes/saline buffer without streptolysin O, resuspended in the same buffer and aliquots of 50 μl [$(1–2) \times 10^6$ cells] were transferred into new Eppendorf tubes and used for the assays.

Abbreviations used: PI3K, phosphoinositide 3-kinase; PI, phosphatidylinositol; PI4P, PI 4-phosphate, PIP_2, PI 4,5-bisphosphate; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; PITP, phosphatidylinositol transfer protein; FMLP, formylmethionyl-leucylphenylalanine; GTP[S], guanosine 5′-[γ-thio]triphosphate; MAPK, mitogen-activated protein kinase; GST, glutathione S-transferase.

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Reconstitution of neutrophils and assay of PIP_2 phosphorylation

The cytosol-depleted cells were incubated with 0.3 nM PI3Kγ and 600 nM recombinant PITP in Pipes/saline for 15 min at room temperature. Recombinant PI3Kγ was expressed as a glutathione S-transferase (GST) fusion protein in Sf9 insect cells and affinity-purified as recently described in [6]. PITPα was purified from bacteria [10]. In control experiments vehicles were used instead of recombinant proteins. Ten-times-concentrated assay buffer was added, giving final concentrations of 3 mM $MgCl_2$, 0.3 mM $CaCl_2$, 1 mM EGTA, 100 μM ATP, 30 μCi of [γ-^{32}P]ATP, 137 mM NaCl, 3 mM KCl and 20 mM Pipes, pH 7.4. The assay buffer contained additionally guanosine 5′-[γ-thio]triphosphate (GTP[S]) and/or FMLP, giving final concentrations of 10 μM (GTP[S]) and 1 μM (FMLP). The final reaction volume was 100 μl. The cell suspension was incubated for 20 min at 37 °C. After incubation the reaction was quenched, lipids were extracted, deacylated, resolved by HPLC, and PIP_3 production was quantified as described previously [6]. As chromatographic standards, [^{32}P]PIP_3 and [^{3}H]inositol 1,3,4,5-tetrakisphosphate were used. Linearity of the time course of PIP_3 production was observed over the duration of the experiments.

Immunoblotting

The loss of PI3Kγ and PITPα from neutrophils was assayed by protein immunoblotting using monoclonal antibodies against PI3Kγ and PITPα. PI3Kγ antibody was raised against GST fusion protein [6], and antibody against PITPα was raised against native recombinant protein. Horseradish peroxidase-conjugated anti-mouse antibody (Sigma, 1:2000 dilution) was used as secondary antibody. Bound peroxidase was revealed by enhanced chemiluminiscence using the ECL (enhanced chemiluminescence) kit from Amersham International.

RESULTS AND DISCUSSION

Permeabilization of human neutrophils with streptolysin O caused the loss of FMLP-induced PI3K activation (Figure 1a), thus confirming recent data [4,5]. The loss of PI3K activity is paralleled by the release of the majority of immunodetectable PI3Kγ and the PITPα into the external medium (Figure 1b). Examination of PI3Kγ and PITP after 40 min permeabilization displayed a small amount of PI3Kγ (but not PITPα) still associated with the cells (results not shown).

Neutrophils depleted of PITP and PI3Kγ were utilized for restoration of FMLP-stimulated PI3Kγ activity. Whereas addition of recombinant PI3Kγ or PITPα alone was unable to induce significant synthesis of PIP_3, in the presence of FMLP or the non-hydrolysable GTP analogue, GTP[S], activation was observed (Table 1). Combined addition of PITPα and PI3Kγ caused a small and variable degree of PIP_3 production which was about 10-fold enhanced in the presence of FMLP and GTP[S]. Table 2 illustrates the effects of FMLP and GTP[S] in the presence and absence of PITP and PI3Kγ. FMLP alone is able to activate PIP_3 production about 5-fold in the presence of added PITP and PI3Kγ. The stimulatory effect of FMLP on PI3K activity in the absence of recombinant PI3Kγ and PITP for reconstitution could be due to some resting activity of endogenous PI3Kγ attached to the permeabilized neutrophils. Pretreatment of neutrophils with pertussis toxin produced about 80 % inhibiton of the stimulatory effect of FMLP and GTP[S] on PIP_3 production in permeabilized cells reconstituted with PI3Kγ and PITP. This finding points to an involvement of G_i proteins in the activation of PI3Kγ.

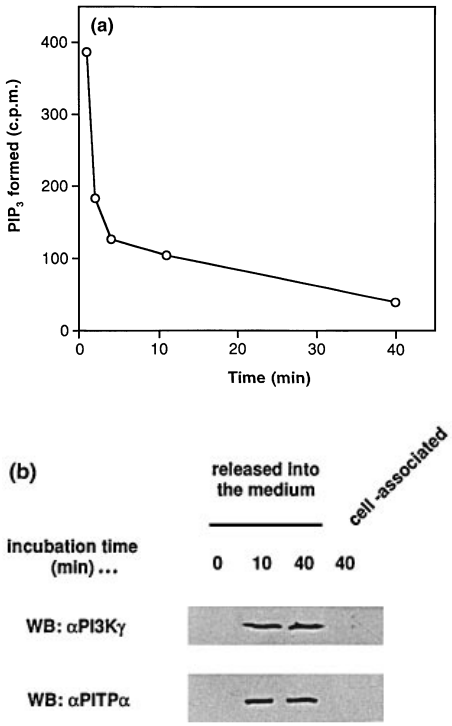

Figure 1 Detection of PI3Kγ in human neutrophils and release of the enzyme on permeabilization with streptolysin O

(**a**) Loss of FMLP/GTP[S]-stimulated PI3K activity on permeabilization with streptolysin O. After treatment with permeabilization cocktail for the indicated time, the cells were incubated for 40 s at 37 °C with assay buffer containing either 10 μM GTP[S] and 1 μM FMLP in a final reaction volume of 100 μl. After incubation the reaction was quenched, lipids were extracted, deacylated, resolved by HPLC and PIP_3 production was assayed. (**b**) Time course of the release of PI3Kγ and PITP from isolated neutrophils. Cells were isolated and permeabilized with streptolysin O. After incubation at 37 °C for the indicated times, the loss of PI3Kγ and PITPα was assayed by protein immunoblotting [6,8] using monoclonal antibodies against PI3Kγ and PITP. WB, Western blot; α, anti-.

Table 1 Reconstitution of PIP_3 production in permeabilized neutrophils with PI3Kγ and PITP: stimulation by FMLP and GTP[S]

Isolated neutrophils were permeabilized with streptolysin O. The cytosol-depleted cells were incubated with 0.3 nM recombinant PI3Kγ and/or 600 nM PITP for 15 min at room temperature. In control experiments vehicles were used instead of recombinant proteins. Assay buffer containing 25–30 μCi of [γ-^{32}P]ATP and either 10 μM GTP[S] or 1 μM FMLP was added to a final reaction volume of 100 μl. The cell suspension was incubated for 20 min at 37 °C and PIP_3 production was assayed as described in the Materials and methods section. Values given are the means ± S.E.M. of three independent observations.

	PIP_3 production (c.p.m.)	
	Without stimulation	With FMLP and GTP[S]
Control	56 ± 8	194 ± 32
PI3Kγ	42 ± 6	472 ± 79
PITP	111 ± 15	417 ± 70
PI3Kγ + PITP	167 ± 24	1667 ± 280

The results of the reconstitution experiments indicate that PI3Kγ can be regulated by the FMLP receptor in cytosol-depleted cells. However, the stimulation of the enzyme is greatly

Table 2 Effects of FMLP and GTP[S] on PIP_3 production in permeabilized neutrophils, reconstituted with PI3Kγ and PITP

Isolated neutrophils were permeabilized with streptolysin O. The cytosol-depleted cells were incubated with 0.3 nM recombinant PI3Kγ and/or 600 nM PITP for 15 min at room temperature. In control experiments vehicles were used instead of recombinant proteins. 1 μM FMLP and/or 10 μM GTP[S] (final concentrations) were added to the permeabilized neutrophils. PIP_3 synthesis was assayed as described above. Values given are the means $\pm$ S.E.M. of three independent observations.

	PIP_3 production (c.p.m.)	
	Without PI3Kγ and PITP	With PI3Kγ and PITP
Control	43 ± 7	85 ± 11
FMLP	234 ± 39	574 ± 80
GTP[S]	106 ± 18	383 ± 54
FMLP + GTP[S]	163 ± 28	1404 ± 235

facilitated by the presence of PITPα. The concentration of endogenous PITP in human neutrophils has been estimated to be about 0.5 μM (A. Ball and S. Cockcroft, unpublished work). This is in the same range as found for the stimulatory effects of the protein, suggesting an important *in vivo* function of PITP during FMLP dependent PIP_3 production. The increase of PIP_3 production observed after addition of GTP[S] to the cells reconstituted with PI3Kγ and PITP could be due to the potency of G_i type of G-proteins to stimulate PI3Kγ activity. GTP[S] is known to stabilize the activated state of heterotrimeric G-proteins [9].

Taken together our data allow a proposal for the mechanism of FMLP-induced PIP_3 production in human neutrophils. After binding to its receptor, FMLP causes an activation of G_i proteins on the cytoplasmic site. Both the activated $G_i\alpha$–GTP and $G\beta\gamma$ subunits are potentially able to stimulate PI3Kγ. A recent study on the involvement of PI3Kγ in the signal transduction of G-protein-coupled receptors to mitogen-activated protein kinase (MAPK) points to an important role of $G\beta\gamma$ subunits in the signalling via PI3Kγ [17].

Synthesis of PIP_3 catalysed by PI3Kγ needs PITP as an essential cofactor. The interplay of both proteins may represent a general mechanism by which the activity of PI3K isoforms is controlled *in vivo*. The molecular mechanism of the functional interaction of PI3Kγ and PITP remains unclear. PITP-dependent transport of PI from intracellular compartments to the plasma membrane seems possible, but the enormous excess of the PI3K substrate PIP_2 over the PI3K product PIP_3 maximally produced in neutrophils [18] makes it questionable whether substrate could be rate-limiting. Thus we favour a more direct involvement of PITP in PIP_2 phosphorylation. Detailed investigations of the effects of PITP on PI3K-dependent PIP_2 phosphorylation *in vitro* are necessary to elucidate this point.

The direct downstream targets of FMLP-stimulated PI3Kγ and its product PIP_3 remain obscure. FMLP regulates a number of enzymic activities in neutrophils, including phospholipase Cβ, Lyn tyrosine kinase and enzymes of the MAPK pathway [19]. Inhibition of FMLP-dependent stimulation of Raf, MAP kinase/Erk kinase ('MEK') kinase and MAPK activities by the PI3K inhibitor wortmannin has been recently demonstrated [20]. In transiently transfected COS-7 cells we identified PI3Kγ as a mediator of G$\beta\gamma$-dependent regulation of MAPK activity [17]. Thus PI3Kγ could function as a direct link between the FMLP receptor and enzymes of the MAPK pathway in neutrophils.

This work was supported by grants from the Deutsche Forschungsgemeinschaft (SFB 197). S.C. thanks the Welcome Trust, the Leukemia Research Fund and the Medical Research Council for their support.

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Received 29 April 1997/16 May 1997; accepted 27 May 1997