Tyrosine phosphorylation-dependent activation of phosphatidylinositide 3-kinase occurs upstream of Ca^{2+}-signalling induced by Fcγ receptor cross-linking in human neutrophils

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The effect of wortmannin on IgG-receptor (FcγR)-mediated stimulation of human neutrophils was investigated. The Ca^{2+} influx induced by clustering of both Fcγ receptors was inhibited by wortmannin, as was the release of Ca^{2+} from intracellular stores. Wortmannin also inhibited, with the same efficacy, the accumulation of Ins(1,4,5)P_3 observed after FcγR stimulation, but did not affect the increase in Ins(1,4,5)P_3 induced by the chemotactic peptide, formyl-methionine-leucine-phenylalanine. Because wortmannin is, in the concentrations used here, an inhibitor of PtdIns 3-kinase, these results suggested a role for PtdIns 3-kinase upstream of Ca^{2+} signalling, induced by FcγR cross-linking. Support for this notion was obtained by investigating the effect of another inhibitor of PtdIns 3-kinase, LY 294002, and by studying the kinetics of PtdIns 3-kinase activation. We found translocation of PtdIns 3-kinase to the plasma membrane and increased PtdIns 3-kinase activity in the membrane as soon as 5 s after FcγR cross-linking, even before the onset of the Ca^{2+} response. Moreover, the translocation of PtdIns 3-kinase to the plasma membrane was inhibited by co-cross-linking of either FcγRIIa and FcγRIIIb with the tyrosine phosphatase, CD45, indicating a requirement for protein tyrosine phosphorylation in the recruitment of PtdIns 3-kinase to the plasma membrane. Taken together, our results suggest a role for PtdIns 3-kinase in early signal transduction events after FcγR cross-linking in human neutrophils.

INTRODUCTION

Human neutrophils possess two types of receptor for IgG (FcγR), FcγRIIa and FcγRIIIb. Each of these receptors induces neutrophil activation after cross-linking. One of the earliest signalling events that is observed is an increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). We have shown that although separate FcγR cross-linking induces release of Ca^{2+} from intracellular stores, cross-linking of both receptors together in a heterotypic complex induces a synergistic Ca^{2+} response, mainly due to an effective stimulation of Ca^{2+} influx from the extracellular medium [1]. As yet, the exact mechanism of neutrophil activation via heterotypic FcγR cross-linking remains to be determined.

FcγRIIIb is linked to the membrane via a glycan–phosphatidylinositol (GPI) anchor. FcγRIIa is a transmembrane receptor containing an immune receptor tyrosine-based activation motif (known as the ITAM or Reth motif) [2] (E-X_8-D-X-X-Y-X-X-L-X_{12}-Y-X-X-L) in the cytoplasmic tail, which has been shown to be involved in signalling of FcγRIIa [3,4]. This signalling motif is found also in subunits of the T- and B-cell antigen receptor complexes [5]. Association of FcγRIIa from neutrophils with the *src*-like kinase Fgr has been described [6]. It is not clear how the GPI-anchored FcγRIIIb, without a transmembrane and cytoplasmic tail, transduces signals through the membrane. It has been suggested that other GPI-anchored molecules use *src*-like kinases for signalling [7]. The mechanism of association between these proteins is probably mediated via lipid–lipid interactions involving the lipid anchor of the receptor and the myristoylation and palmitoylation of the *src*-like kinase [8].

The lipid kinase, PtdIns 3-kinase, which has been found in many activated receptor complexes [9,10], phosphorylates PtdIns, PtdIns4*P* and PtdIns(4,5)P_2 to form PtdIns3*P*, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 respectively. It has been suggested that these lipids act as second messengers [11], but their functions are not yet clear. PtdIns 3-kinase is a heterodimeric protein containing an 85 kDa regulatory subunit (p85) and a 110 kDa catalytic subunit (p110) [12]. The p85 subunit contains an SH2 domain that can bind to tyrosine-phosphorylated proteins in the membrane, inducing activation of the enzyme [13], e.g. during T-cell receptor activation to the phosphorylated immune receptor tyrosine-based activation motif of CD3ζ [14]. Recently, a role for PtdIns 3-kinase in FcγR-induced signalling has been suggested. In guinea pig neutrophils, antibody-dependent cellular cytotoxicity is inhibited by wortmannin, an inhibitor that appears to be specific for PtdIns 3-kinase in the nanomolar range [15–18]. Triggering of either FcγRI or FcγRII in U937 cells induces increased PtdIns 3-kinase activity [19].

The results of our study suggest the involvement of PtdIns 3-kinase in FcγR-induced Ca^{2+} signalling in human neutrophils. To investigate the relevance of tyrosine phosphorylation in this process, we have studied the effect of bringing CD45, a membrane-bound protein tyrosine phosphatase [20], which is highly expressed on neutrophils, in close proximity to the FcγR.

Abbreviations used: bsAb, bispecific antibody; FcγR, the receptors for IgG; GAM, goat anti-mouse; PtdIns 3-kinase, 1-phosphatidylinositol 3-kinase; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; fMLP, formyl-methionine-leucine-phenylalanine; GPI, glycan–phosphatidylinositol; mAb, monoclonal antibody.

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MATERIALS AND METHODS

Materials

Wortmannin, formyl-methionine-leucine-phenylalanine (fMLP) and streptavidin were from Sigma Chemical Co. (St. Louis, MO, U.S.A.), ionomycin from Calbiochem (La Jolla, CA, U.S.A.) and indo-1/AM from Molecular Probes (Eugene, OR, U.S.A.). LY 294002 was from Biomol (Plymouth Meeting, PA, U.S.A.). Wortmannin was dissolved in DMSO at a concentration of 100 μM. LY 294002 was dissolved in DMSO at a concentration of 80 mM. $F(ab')_2$ fragments of polyclonal goat anti-mouse (GAM) IgG, Fcγ fragment specific [GAM-Fc $F(ab')_2$] and $F(ab')_2$ fragments of polyclonal GAM IgG [GAM $F(ab')_2$] were purchased from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA, U.S.A.). The incubation medium contained 132 mM NaCl/6 mM KCl/1 mM $CaCl_2$/1 mM $MgSO_4$/1.2 mM potassium phosphate/20 mM Hepes/5.5 mM glucose/0.5 % (w/v) human serum albumin, pH 7.4.

Isolation of neutrophils

Neutrophils from blood obtained from healthy individuals were isolated by Percoll density gradient centrifugation and isotonic lysis of contaminating erythrocytes as described previously [1].

Antibodies

The anti-FcγRIII monoclonal antibodies (mAbs) were 3G8 (mIgG1) [21] and CLBFcgran1 (mIgG2a) [22]. CLBFcgran 1 was used in all experiments except for the control responses for the bispecific antibody (bsAb). The anti-(human FcγRII) mAb IV.3 (mIgG2b) [23] was used. As CD45 antibody, mAb 3D3 (mIgG1) was used [R. A. W. van Lier, Central Laboratory of the Netherland's Red Cross Blood Transfusion Service (CLB), Amsterdam, The Netherlands]. W6/32 mAb, directed against HLA class I, was used as a control antibody. The mAbs were purified from hybridoma culture supernatant by precipitation with 50 % saturated ammonium sulphate and subsequent Protein A affinity chromatography. Fab fragments were prepared by digestion with 4 % (w/v) papain in PBS containing 5 mM EDTA and 10 mM cysteine, for 90 min at 37 °C. The reaction was terminated by adding 20 mM iodoacetamide. $F(ab')_2$ fragments were prepared by digestion with 2 % (w/v) pepsin, pH 3.7, for 16 h at 37 °C. Fc parts were removed by Protein A affinity-chromatography. Antibodies were biotinylated with *N*-hydroxy-succinimide-LC-biotin (250 μg/ml) (Pierce, Rockfort, IL, U.S.A.) for 4 h at room temperature. Free biotin was removed by dialysis against PBS.

Cell activation

Heterotypic FcγR cross-linking was performed by preincubation of the cells with biotinylated Fab fragments of anti-FcγRII and anti-FcγRIII mAbs (5 μg/ml each) for 5 min at 37 °C. Cells were washed and resuspended in incubation medium, prewarmed to 37 °C. The cells were incubated for 5 min at 37 °C in the absence or presence of wortmannin or LY 294002. Cross-linking of biotinylated antibodies was performed by adding streptavidin (1.5 μg/ml or 10 μg/ml, as specified in the Figure legends), with stirring. Co-cross-linking of FcγRs with CD45 was performed by preincubation of the cells with biotinylated $F(ab')_2$ fragments of CD45 (5 μg/ml) for 5 min at 37 °C. Subsequently, biotinylated Fab fragments of anti-FcγRII (5 μg/ml) or anti-FcγRIII mAb (5 μg/ml) or both together were added. After 5 min at 37 °C, cells were washed and resuspended in incubation medium that had been prewarmed to 37 °C. Cross-linking was performed with streptavidin (10 μg/ml) with stirring. Alternatively, neutrophils were pretreated for 5 min at 37 °C with a bsAb (see below) directed against FcγRIII and CD45 (20 μg/ml) or with anti-FcγRIII Fab fragments (10 μg/ml). Cross-linking was performed with GAM $F(ab')_2$ fragments (50 μg/ml).

Preparation of bispecific antibody

The bsAb directed against FcγRIII and CD45 was prepared by chemical cross-linking. 3G8 mAb and 3D3 mAb were digested by pepsin to $F(ab')_2$ fragments. 3G8 $F(ab')_2$ and 3D3 $F(ab')_2$ were reduced with 15 mM 2-mercaptoethanol in PBS/2 mM EDTA for 30 min at 30 °C to obtain Fab′SH fragments. Excess 2-mercaptoethanol was removed by gel filtration in a Sephadex-G25 column equilibrated with PBS/2 mM EDTA, pH 6.7. 3G8 Fab′SH was incubated with a $40\times$ molar excess of bis-maleimidohexane (Pierce) for 60 min at 30 °C. Free bis-maleimidohexane was removed by gel filtration in a Sephadex-G25 column equilibrated with PBS/2 mM EDTA, pH 6.7. 3G8 Fab–bis-maleimidohexane and 3D3 Fab′SH were concentrated to approx. 5 mg/ml in C30 Amicon concentration units (Amicon, Beverly, MA, U.S.A.) and were incubated together for 16 h at room temperature. The yield of bsAb using this procedure was about 30 %. The bsAb was separated from the remaining Fab fragments by gel filtration by FPLC in a Superose 12 column equilibrated with PBS.

Characterization of the bsAb, FcγRIII $\times$ CD45

Binding of the bsAb to various cells was analysed by indirect immunofluorescence and flow cytometry (FACScan, Becton Dickinson, San Jose, CA, U.S.A.). As expected, the bsAb, FcγRIII $\times$ CD45, showed a high degree of binding to neutrophils (results not shown). The bsAb also showed binding to the EBV-transformed B-cell line, JY, which expresses CD45 but lacks FcγRIII: a mean fluorescence intensity of 566 ± 59 was obtained, compared with 67 ± 6 with the anti-FcγRIII $F(ab')_2$, 517 ± 55 with CD45 $F(ab')_2$ and 61 ± 5 with the isotype-matched control (means $\pm$ S.E.M. of three experiments). In addition, the bsAb showed binding to $CHO^{Fc\gamma RIIIb}$ cells that were transfected with FcγRIIIb [24] but expressed no CD45; a mean fluorescence intensity of 737 ± 71 was obtained compared with 552 ± 69 with anti-FcγRIII $F(ab')_2$, 98 ± 7 with anti-CD45 $F(ab')_2$ and 93 ± 4 with the isotype-matched control (means $\pm$ S.E.M. of three experiments). Untransfected CHO^{wt} cells did not bind the bsAb. These experiments showed that both antigen recognition sites were present in the bsAb, FcγRIII $\times$ CD45.

Measurements of the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$)

Determination of $[Ca^{2+}]_i$ was performed as described previously [1]. In experiments carried out in the presence of EGTA, cells were preincubated with biotinylated antibodies in normal incubation medium, washed and resuspended at a concentration of 2×10^6 cells/ml in medium without Ca^{2+}. Just before cross-linking, 1 mM EGTA was added. Assessment of Ca^{2+} influx was carried out using the Mn^{2+} quenching technique, as described previously [1].

Measurement of Ins(1,4,5)P_3 levels

Ins(1,4,5)P_3 was assayed using a system from Amersham International. Neutrophils (1×10^7 cells/ml in incubation medium) were preincubated with biotinylated anti-FcγR Fab fragments

for 5 min, washed and resuspended in PBS [Ins(1,4,5)P_3 levels could not be measured reliably when incubation medium was used, probably due to breakdown after disruption of the cells]. At various times after stimulation of the cells with streptavidin, 300 μl of each sample was added to 150 μl of 50 mM Tris/HCl (pH 8.0) containing 4 mM EDTA, frozen immediately in liquid N_2 and stored at -80 °C. After thawing, the samples were centrifuged in the cold for 5 min at 10000 ***g***. Ins(1,4,5)P_3 was assessed by measuring the competition between unlabelled Ins(1,4,5)P_3, present in the samples, and a fixed amount of [^{3}H]Ins(1,4,5)P_3 for a limited number of binding sites on an Ins(1,4,5)P_3-binding protein preparation. The amount of Ins(1,4,5)P_3 was determined by intrapolation from a standard curve obtained with known quantities of unlabelled Ins(1,4,5)P_3.

Detection of PtdIns 3-kinase translocation

Fractionation of membranes and cytosol from neutrophils was performed as described previously [25]. Neutrophils (1×10^7 cells/ml in incubation medium) were preincubated for 5 min at 37 °C with biotinylated Fab fragments of anti-FcγRII mAb and anti-FcγRIII mAb. The cells were washed and resuspended in incubation medium at 37 °C and were transferred to a stirred cuvette maintained at 37 °C. Cross-linking was performed by the addition of streptavidin. At different time points, stimulation was stopped by the addition of a 5× excess of ice-cold PBS. The neutrophils were centrifuged and resuspended (8×10^7 cells/ml) in ice-cold sonication buffer [10 mM Hepes, pH 7.2/1 mM EGTA/5 % (w/v) sucrose, containing a protease inhibitor cocktail (Complete™, Boehringer, Mannheim, Germany)]. After sonication of the neutrophil suspension (3×15 s at 21 kHz frequency and 8 μm peak-to-peak amplitude), 1 ml of postnuclear supernatant was layered on to a discontinuous sucrose gradient, consisting of 1 ml of 52 % (w/v) sucrose, 1 ml of 40 % (w/v) sucrose and 1 ml of 15 % (w/v) sucrose. After centrifugation (100000 ***g***, for 35 min at 4 °C), 80 % of the supernatant (cytosol) and the interface between the 15 % and 40 % sucrose layers (plasma membranes) were collected. Membrane fractions were treated with ice-cold trichloroacetic acid (10 %, w/v) for 15 min. After centrifugation (12000 ***g***, for 15 min at 4 °C), pellets were taken up in Laemmli sample buffer [125 mM Tris/HCl, pH 6.8 containing 8 % (w/v) SDS, 10 % (v/v) 2-mercaptoethanol and 10 % (v/v) glycerol] and were boiled for 5 min. The presence of PtdIns 3-kinase in the cytosol fraction (derived from 8×10^5 cells) and the plasma membrane fraction (derived from 4×10^7 cells) was determined by SDS/PAGE and immunoblotting with a polyclonal rabbit antibody directed against the 85-kDa subunit (p85) of PtdIns 3-kinase (Affinity, Nottingham, U.K.). For detection of the primary antibody, the blots were incubated with horse anti-rabbit immunoglobulin antibodies conjugated to horseradish peroxidase (CLB, Amsterdam, The Netherlands) and were developed using enhanced chemiluminescence (Boehringer). The proteins were visualized by exposure to a KODAK X-OMAT S film for 1 min.

Measurement of PtdIns 3-kinase activity

An equal volume of lysis buffer [6 % (v/v) Nonidet P-40, 40 mM Tris/HCl, pH 8.0, 200 mM NaCl, 20 mM $Na_3P_2O_7$, 4 mM EDTA, 100 mM NaF, 2 mM Na_3VO_4, 2 mM PMSF, 200 μM leupeptin, 8 μg/ml soybean trypsin inhibitor, 20 μg/ml aprotinin] was added to the neutrophil membrane and cytosol fractions (25×10^6 cell and 12.5×10^6 cell equivalents per immunoprecipitation respectively). A lysate of rat-1 fibroblasts served as a positive control for the measurement of PtdIns 3-kinase activity. Lysates were immunoprecipitated for 1 h at 4 °C with anti-p85 polyclonal rabbit antiserum (UBI, Lake Placid, NY, U.S.A.) or normal rabbit serum (1.7 μl per sample). Protein A–Sepharose (Sigma) was added before incubation overnight at 4 °C. The Protein A–Sepharose beads were washed four times with half-strength lysis buffer and twice with 10 mM Tris/HCl, pH 7.4, containing 1 mM Na_3VO_4. PtdIns 3-kinase activity was measured by adding 100 μg of sonicated PtdIns (Sigma) and 10 μCi of [γ-^{32}P]ATP in the presence of 200 μM adenosine, 30 mM $MgCl_2$ and 40 mM ATP in a volume of 60 μl. The reactions were carried out for 20 min at room temperature and were stopped by the addition of 100 μl of 1 M HCl and 200 μl of chloroform/methanol (1:1, v/v). After centrifugation and removal of the upper layer, 80 μl of methanol/HCl was added and the mixture was again centrifuged. The lipids were separated on TLC plates (Merck) using a solvent system of chloroform/methanol/N-H_4OH (45:35:10, by vol.). The TLC plates were exposed to X-ray film at -80 °C.

Western blot analysis for phosphotyrosine

Neutrophils were suspended in incubation medium (as described above) without human serum albumin. The cells (10^7 cells/ml) were preincubated at 37 °C with biotinylated mAb as indicated in the text, washed and resuspended at 10^7 cells/ml. At various times after the addition of streptavidin, 50 μl of cell suspension was mixed with 50 μl of sample buffer [125 mM Tris/HCl, pH 6.8, 4 % (w/v) SDS, 10 % (v/v) 2-mercaptoethanol and 10 % (v/v) glycerol] containing 1 mM Na_3VO_4, preheated to 100 °C and maintained at 100 °C for 10 min. The samples were analysed after SDS/PAGE by immunoblotting with monoclonal anti-phosphotyrosine antibody (UBI). The amount of anti-phosphotyrosine bound to the blot was determined with peroxidase-labelled horse anti-mouse Ig and enhanced chemiluminescence.To verify equal loading of protein in each lane, blots were subsequently incubated with a rabbit antiserum directed against the C-terminus of p47-*phox* [25], which was detected by peroxidase-labelled horse anti-rabbit Ig antibodies (CLB, Amsterdam, The Netherlands).

Statistical analysis

For statistical analysis, paired Student's *t*-tests were performed. *P* values exceeding 0.05 were considered not significant.

RESULTS

Effect of wortmannin on FcγR-induced Ca^{2+} responses

In a previous study we observed that the heterotypic clustering of both FcγRs on human neutrophils primarily induces Ca^{2+} influx from the extracellular medium [1]. To obtain more insight into the signalling pathways involved, we investigated the effect of wortmannin. Wortmannin has been shown to interfere with a protein kinase C-independent pathway necessary for fMLP-induced superoxide production [26] and has subsequently been shown to inhibit the activity of PtdIns 3-kinase [15,17]. Ca^{2+} influx induced by cross-linking of both FcγRIIa and FcγRIIIb, as measured by Mn^{2+} influx, was completely inhibited by 100 nM wortmannin (Figure 1, upper panel).

In neutrophils, the capacitive Ca^{2+} entry model, in which depletion of the Ca^{2+} stores generates a signal that induces Ca^{2+} influx from the extracellular medium, seems applicable [27,28]. To investigate whether the site of action of wortmannin was upstream or downstream of the mobilization of Ca^{2+} from the stores, FcγR cross-linking was performed in the presence of EGTA to prevent Ca^{2+} influx. Under these conditions, the

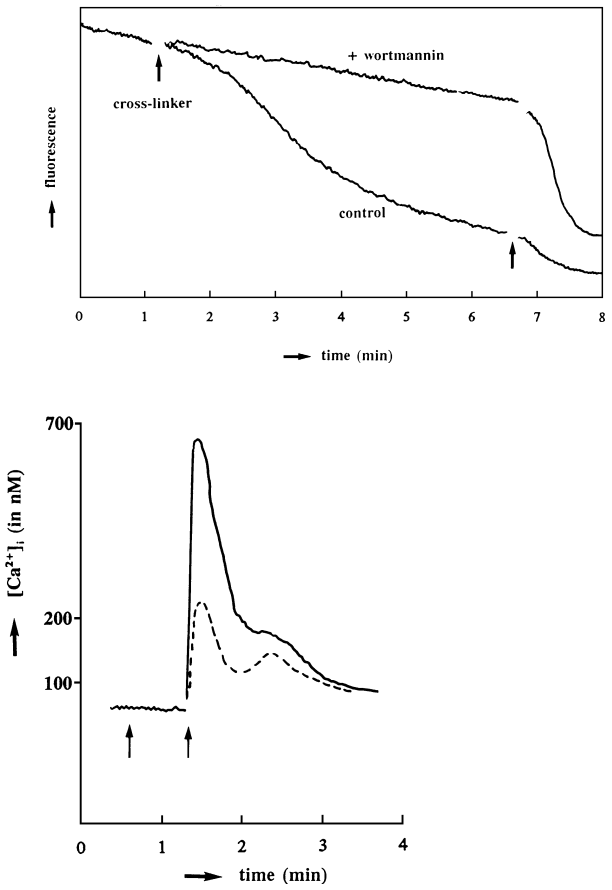

Figure 1 Effect of wortmannin on Ca^{2+} influx and intracellular Ca^{2+} mobilization induced as a result of heterotypic FcγR cross-linking

Indo-1-loaded neutrophils preincubated with biotinylated Fab fragments of anti-FcγR mAb were resuspended in incubation medium (upper panel) with 0.2 mM Ca^{2+} or medium without Ca^{2+} (lower panel). Cells were treated with or without (control) 100 nM wortmannin. For measurement of Ca^{2+} influx (upper panel), 0.5 mM $MnCl_2$ was added 2 min before the addition of streptavidin (10 μg/ml, first arrow). To completely quench Mn^{2+}-induced indo-1 fluorescence, digitonin (10 μM) was added (second arrow). The results shown are representative of three experiments. For measurement of Ca^{2+} mobilization (lower panel), 1 mM EGTA (first arrow) was added 30 s before the addition of streptavidin (10 μg/ml, second arrow). The mean change in peak values of $[Ca^{2+}]_i$ were (means $\pm$ S.E.M., $n = 4$) 644 ± 116 nM in the absence of wortmannin and 112 ± 20 nM in the presence of 100 nM wortmannin. The Ca^{2+} concentration in resting cells was 60 ± 2 nM.

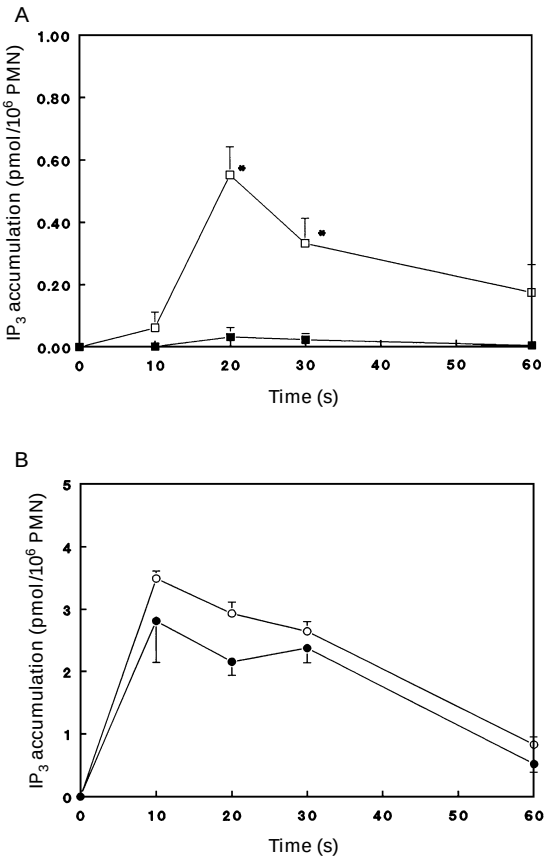

Figure 2 Effect of wortmannin on the Ins(1,4,5)P_3 accumulation induced after (A) heterotypic FcγR cross-linking or (B) fMLP stimulation

(**A**) Neutrophils (PMN) were preincubated with biotinylated Fab fragments of anti-FcγR mAb. The cells were incubated with (■) or without (□) 100 nM wortmannin; cross-linking was performed by the addition of 1.5 μg/ml streptavidin. Samples for Ins(1,4,5)P_3 (IP_3) measurement were taken at different time points as described in the Materials and methods section. (**B**) For comparison, untreated cells were incubated with (●) or without (○) 100 nM wortmannin and stimulated with 1 μM fMLP. At the time-points marked with an asterisk, wortmannin inhibited the Ins(1,4,5)P_3 (IP_3) accumulation significantly ($P < 0.05$). Values are the means $\pm$ S.E.M. of three independent experiments.

Ca^{2+} response induced by heterotypic FcγR cross-linking was also inhibited by wortmannin (Figure 1, lower panel). Half-maximal inhibition was observed at a concentration of approximately 25 nM wortmannin (results not shown).

Effect of wortmannin on FcγR-induced Ins(1,4,5)P_3 accumulation

Under our experimental conditions, the heterotypic cross-linking of FcγR released Ca^{2+} from intracellular stores. This probably results from the generation of Ins(1,4,5)P_3 [29]. Measurements of the levels of Ins(1,4,5)P_3 indeed showed a clear increase upon heterotypic FcγR cross-linking (Figure 2A), suggesting that the Ca^{2+} response is (at least in part) mediated by a rise in InsP_3. The increase in the Ins(1,4,5)P_3 accumulation after FcγR cross-linking was also inhibited by wortmannin (Figure 2A). For comparison, the fMLP-induced generation of Ins(1,4,5)P_3 was also measured in these experiments and found to be unaffected by wortmannin (Figure 2B). This latter finding is in accordance with previous data showing that the Ca^{2+} response evoked by fMLP is not sensitive to wortmannin [26]. The difference in sensitivity to wortmannin is not due to the different amounts of Ins(1,4,5)P_3 accumulation. Higher concentrations of streptavidin (10 μg/ml) induced an increase in the accumulation of Ins(1,4,5)P_3 of 3.24 ± 0.92 pmol/10^6 cells at 20 s after stimulation. In the presence of wortmannin, this accumulation was inhibited by 95 % (to 0.14 ± 0.16 pmol/10^6 cells).

The IC_{50} value for the wortmannin inhibition of Ins(1,4,5)P_3 accumulation after FcγR cross-linking (under conditions of cross-linking with 10 μg/ml streptavidin) was approx. 25 nM (results not shown), which is comparable with the wortmannin concentration needed for inhibition of the Ca^{2+} response under these conditions. In addition, the accumulation of Ins(1,4,5)P_3 upon heterotypic FcγR cross-linking was also inhibited by another inhibitor of PtdIns 3-kinase, LY 294002 [30]. The IC_{50} for LY 294002 was approx. 1.5 μM (results not shown).

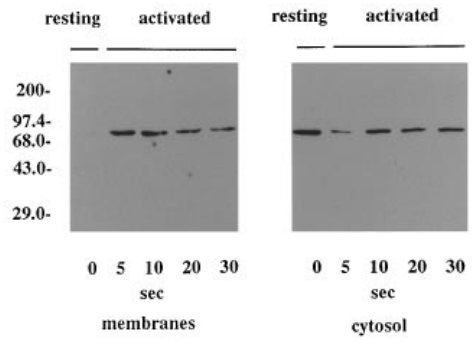

Figure 3 Effect of heterotypic FcγR cross-linking on the localization of PtdIns 3-kinase

Neutrophils preincubated with biotinylated Fab fragments of anti-FcγR mAb were activated by addition of 1.5 μg/ml streptavidin. Under resting conditions, no streptavidin was added. At different time points neutrophils were fractionated as described in the Materials and methods section. Immunoblotting with membrane and cytosol fractions was performed with an antibody directed against the p85 subunit of PtdIns 3-kinase. This Figure is representative of three independent experiments. Molecular-mass markers (kDa) are shown on the left.

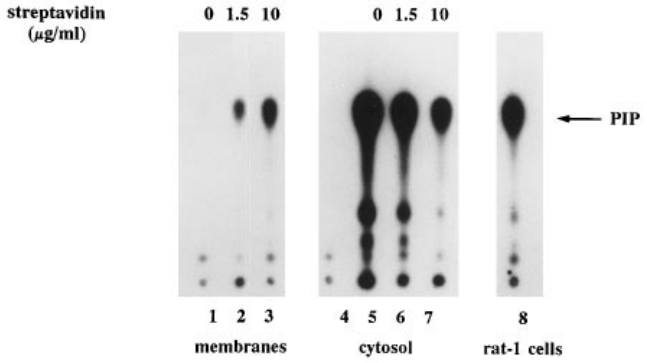

Figure 4 Effect of heterotypic FcγR cross-linking on the activation of PtdIns 3-kinase

Neutrophils were preincubated with biotinylated Fab fragments of anti-FcγR mAb and activation by addition of 1.5 μg/ml (lanes 2 and 6) or 10 μg/ml (lanes 3 and 7) streptavidin. Under resting conditions, no streptavidin was added (lanes 1 and 5). After 5 s stimulation, samples were taken as described in the Materials and methods section. Immunoprecipitations of p85 (all lanes except lane 4) or normal rabbit serum (lane 4) from membrane and cytosol fractions were assayed for kinase activity towards PtdIns. The location of PtdIns3*P* (PIP) in the chromatogram was confirmed by comparison with PtdIns3*P* generated enzymically by immunoprecipitation of p85 from Rat-1 fibroblasts. This Figure is representative of three independent experiments.

Localization and activity of PtdIns 3-kinase

Because early signalling events after FcγR cross-linking appeared sensitive to wortmannin and LY 294002, we investigated the possibility that PtdIns 3-kinase is activated very early after Fcγ receptor activation in human neutrophils. We observed an increased amount of the p85 subunit of PtdIns 3-kinase in the membrane after only 5 s (Figure 3), before any increase in Ins(1,4,5)P_3 was observed under these suboptimal conditions of cross-linking (Figure 2A). Neither biotinylated Fab fragments of anti-FcγR mAb nor streptavidin alone induced translocation of p85 to the membrane (results not shown). We did not always observe a clear decrease in the amount of p85 in the cytosol fraction, probably due to the relatively small amount of PtdIns 3-kinase translocating to the membrane. The Ca^{2+} ionophore, ionomycin, did not induce translocation of p85 to the membrane (results not shown) indicating that the FcγR-induced translocation was not the consequence of the Ca^{2+} elevation observed.

To establish that the p85 subunit of PtdIns 3-kinase recovered in the membrane fraction was part of an active enzyme complex, the activity of PtdIns 3-kinase was measured in membrane and cytosol fractions after FcγR cross-linking. Increased PtdIns 3-kinase activity in the membrane was observed after 5 s of heterotypic FcγR cross-linking (Figure 4). Even under conditions of suboptimal cross-linking using 1.5 μg/ml streptavidin, before any increase in Ins(1,4,5)P_3 accumulation was detected (Figure 2A), an increased PtdIns 3-kinase activity was observed. In addition, a decrease in PtdIns 3-kinase activity was found in the cytosol after FcγR cross-linking.

Table 1 Effect of CD45 co-cross-linking on the FcγR-induced increase in $[Ca^{2+}]_i$ in human neutrophils

Indo-1-loaded neutrophils were preincubated with the indicated antibodies as described in the Materials and methods section. The peak increase in $[Ca^{2+}]_i$ after cross-linking represents the means ± S.E.M. of 3–4 experiments. $[Ca^{2+}]_i$ in resting cells amounted to 62 ± 1 nM ($n = 36$). *Significantly different from control response ($P < 0.05$).

Treatment	Increase in $[Ca^{2+}]_i$ (nM)
Anti-FcγRII biotinylated Fab + streptavidin	709 ± 102
Anti-FcγRII + CD45 biotinylated F(ab′)$_2$ + streptavidin	7 ± 7
Anti-FcγRIII biotinylated Fab + streptavidin	511 ± 102
Anti-FcγRIII + CD45 biotinylated F(ab′)$_2$ + streptavidin	99 ± 30*
Anti-FcγRII biotinylated Fab + anti-FcγRIII biotinylated Fab + streptavidin	2646 ± 416
Anti-FcγRII biotinylated Fab + anti-FcγRIII biotinylated Fab + CD45 biotinylated F(ab′)$_2$ + streptavidin	1202 ± 119*
Anti-FcγRIII Fab + GAM F(ab′)$_2$	116 ± 47*
BsAb FcγRIII × CD45 + GAM F(ab′)$_2$	10 ± 1*
CD45 F(ab′)$_2$ + anti-FcγRIII Fab + GAM F(ab′)$_2$	2 ± 2*

Inhibition of the FcγR-induced tyrosine phosphorylation and translocation of PtdIns 3-kinase by CD45

In order to investigate the role of tyrosine phosphorylation in the FcγR-induced translocation of PtdIns 3-kinase in human neutrophils, the protein tyrosine phosphatase, CD45, was co-cross-linked with FcγRs. As observed by Hoffmeyer et al. [31], co-cross-linking of CD45 with FcγRIIIb or FcγRIIa strongly inhibited the FcγR-mediated Ca^{2+} response (Table 1). The inhibitory effects of co-cross-linking appeared to be specific for CD45, because co-cross-linking of FcγRIIIb with another surface molecule, MHC class I, of these cells [by cross-linking biotinylated CLBFcgran 1 F(ab′)$_2$ and biotinylated W6/32 F(ab′)$_2$ with streptavidin] did not affect the Ca^{2+} response caused by FcγRIIIb cross-linking (results not shown). The Ca^{2+} response induced by heterotypic cross-linking of both FcγRIIa and FcγRIIIb was inhibited by co-cross-linking of CD45, but to a lesser extent than that induced by FcγR cross-linking alone (Table 1).

Additional experiments were performed with a bsAb, able to bind to both FcγRIII and CD45. Treatment of neutrophils with the bsAb FcγRIII × CD45 followed by cross-linking with GAM F(ab′)$_2$ induced only a slight increase in $[Ca^{2+}]_i$, which was 10 % of the control response observed after cross-linking of anti-FcγRIII Fab with GAM F(ab′)$_2$ (Table 1). Co-cross-linking of CD45 together with anti-FcγRIIIb Fab fragments also abolished

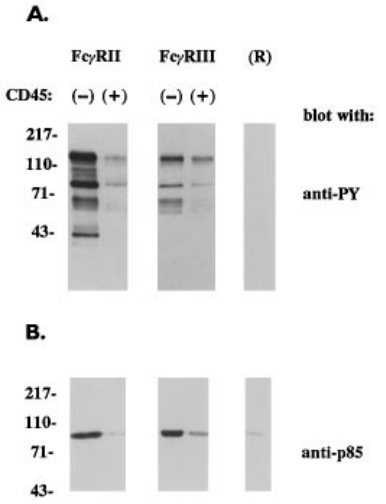

Figure 5 Effect of co-cross-linking of CD45 with FcγRs on (A) tyrosine phosphorylation and (B) translocation of PtdIns 3-kinase to the membrane

Neutrophils were pretreated with biotinylated anti-FcγR Fab (lanes 1 and 3, lanes are numbered from the left) or biotinylated CD45 $F(ab')_2$ and biotinylated anti-FcγR Fab (lanes 2 and 4). Samples were removed 10 s after cross-linking with streptavidin (10 μg/ml). As control, only cross-linker was added (lane R). Western blotting was performed (**A**) with anti-phosphotyrosine (PY) on neutrophil lysates or (**B**) with rabbit antiserum directed against the p85 subunit of PtdIns 3-kinase (anti-p85) in membrane fractions of neutrophils. Similar results were obtained in three experiments.

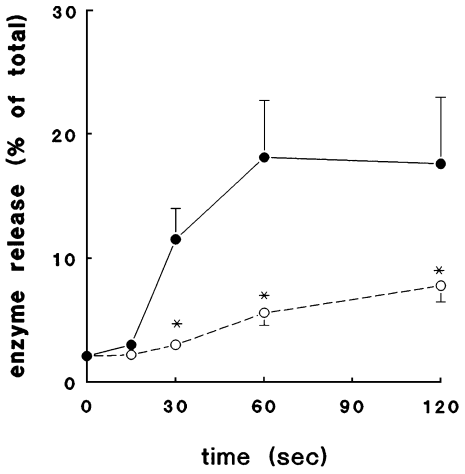

Figure 6 Effect of co-cross-linking of CD45 with FcγRIIIb on the FcγRIIIb-induced release of β-hexosaminidase

Neutrophils were preincubated without (●) or with (○) biotinylated CD45 $F(ab')_2$ and then with biotinylated anti-FcγRIII Fab fragments. After washing the cells, streptavidin (10 μg/ml) was used to cross-link bound antibodies. Results shown are the means ± S.E.M. of three experiments. *Significantly different from control ($P < 0.05$).

the Ca^{2+} response in these experiments (Table 1). The control response was lower than in the other experiments depicted in Table 1, because the anti-FcγRIII Fab fragments were cross-linked with GAM $F(ab')_2$, which gave consistently lower responses than with biotin–streptavidin.

We next investigated the effect of CD45 co-cross-linking on protein tyrosine phosphorylation upon FcγR-induced activation. Treatment of human neutrophils with biotinylated Fab fragments of anti-FcγRIIa alone did not induce an increase in protein tyrosine phosphorylation (results not shown), but when this antibody was cross-linked with streptavidin, a clear increase in protein tyrosine phosphorylation was induced (Figure 5A). Streptavidin alone did not increase protein phosphorylation. After 10 s, the extent of protein tyrosine phosphorylation decreased again (results not shown). Stimulating neutrophils by cross-linking of FcγRIIIb also induced protein tyrosine phosphorylation, with some differences in the pattern when compared with FcγRIIIb cross-linking (Figure 5A). Tyrosine phosphorylation under these conditions was maximal 30 s after cross-linking (results not shown). Co-cross-linking of CD45 in order to introduce CD45 into the FcγR complexes, inhibited both the FcγRIIa-and the FcγRIIIb-induced protein tyrosine phosphorylation (Figure 5A). In accordance with the lower efficacy of CD45 co-cross-linking in inhibiting the Ca^{2+} response after heterotypic cross-linking of FcγRIIa and FcγRIIIb (Table 1), the effect of co-cross-linking of CD45 on tyrosine phosphorylation proved to be less pronounced than its effect on the separate FcγR responses (results not shown).

The inhibition of tyrosine phosphorylation by co-cross-linking of FcγR with CD45 was then used to investigate the role of protein tyrosine phosphorylation in the translocation of PtdIns 3-kinase. Both cross-linking of FcγRIIa and of FcγRIIIb resulted in translocation of PtdIns 3-kinase to the membrane fraction (Figure 5B). The translocation after cross-linking of FcγRIIIb was diminished when CD45 was co-cross-linked with FcγRIIIb (Figure 5B), whereas the FcγRIIa-induced translocation of PtdIns 3-kinase was almost completely prevented by co-cross-linking of CD45 (Figure 5B). The inhibition of PtdIns 3-kinase translocation upon FcγRIIIb ligation by CD45 co-cross-linking was largely prevented by pretreatment of the cells with the tyrosine phosphatase inhibitor pervanadate (25 μM) (results not shown).

Inhibition of FcγR-induced degranulation

We also investigated the effect of CD45 and wortmannin on functional responses of neutrophils induced via FcγR cross-linking. Cross-linking of FcγRIIa hardly induced any release of β-hexosaminidase (results not shown). Cross-linking of FcγRIIIb in cytochalasin B-treated neutrophils evoked within 30 s the release of β-hexosaminidase (Figure 6), an enzyme present in azurophilic granules [32], and vitamin B_{12}-binding protein (results not shown), a marker for specific granules [33]. In the absence of cytochalasin B, virtually no granule proteins were detected in the supernatant (results not shown). Upon cross-linking of CD45 into the FcγRIIIb complex, the release of β-hexosaminidase was abolished (Figure 6) at early time-points. At later times there was some increase in the amount of β-hexosaminidase in the supernatant, indicating that the process of degranulation was delayed under these conditions. A similar pattern of inhibition was observed for the release of vitamin B_{12}-binding protein (results not shown). In addition, we also found almost complete inhibition of both FcγRIIIb- and heterotypic FcγR-induced release of β-hexosaminidase by 100 nM wortmannin: a release of 88 % of the total amount of β-hexosaminidase for heterotypic FcγR activation was only 9 % in the presence of wortmannin and a release of 34 % of the total amount of β-hexosaminidase for FcγRIIIb activation was only 6 % in the presence of wortmannin.

DISCUSSION

The results of this study indicate that FcγR-induced Ca^{2+} signalling in human neutrophils involves the activation of PtdIns

3-kinase at a step prior to the generation of Ins(1,4,5)P_3. The evidence for this notion comes from two directions. Firstly, we observed inhibition of Ins(1,4,5)P_3 production, Ca^{2+} mobilization and influx after heterotypic FcγR cross-linking by wortmannin, an inhibitor of PtdIns 3-kinase [15,18], with an IC_{50} value of about 25 nM. This concentration is somewhat higher than the concentration of wortmannin required to inhibit the purified PtdIns 3-kinase [34], but similar to the wortmannin concentration required for treatment of intact cells to inhibit PtdIns 3-kinase activity recovered in the membrane fraction after FcγR cross-linking (results not shown). In addition, another specific PtdIns 3-kinase inhibitor, LY 294002, also inhibited the accumulation of Ins(1,4,5)P_3 upon heterotypic FcγR cross-linking with an IC_{50} of approximately 1.5 μM, which is comparable with an IC_{50} of 2.5 μM to inhibit fMLP-induced superoxide production in neutrophils [35] and a IC_{50} of 1.4 μM against purified PtdIns 3-kinase [30]. PtdIns 4-kinase, the enzyme involved in the generation of PtdIns(4,5)P_2, has been reported not to be inhibited by treatment of intact cells with wortmannin at concentrations up to 300 nM [36] or by LY 294002 [30], suggesting that the inhibition of the Ins(1,4,5)P_3 accumulation by these inhibitors was not due to an inhibition of PtdIns 4-kinase. It should also be noted that, in neutrophils, wortmannin was without effect on fMLP-induced Ins(1,4,5)P_3 generation (Figure 2B), making an effect on PtdIns metabolism as an alternative explanation highly unlikely.

A second observation indicating the involvement of PtdIns 3-kinase in FcγR signalling in human neutrophils is the very early activation of this enzyme. When suboptimal cross-linking of FcγR was induced, translocation of PtdIns 3-kinase occurred before the increase in Ins(1,4,5)P_3 (Figures 2A and 3). An increase in PtdIns 3-kinase activity in the membrane was observed also very soon after activation (Figure 4), indicating that, in addition to the presence of the p85 subunit of PtdIns 3-kinase, p110 (the catalytic subunit) translocates after FcγR cross-linking. In human neutrophils, there is a suggestion of an alternative PtdIns 3-kinase activity [37]. This enzyme is activated via G-proteins and also inhibited by wortmannin. Our observations, indicating abolishment of both the FcγR-induced protein tyrosine phosphorylation and the translocation of PtdIns 3-kinase by co-cross-linking the tyrosine phosphatase CD45 into the FcγR complexes, suggest a role for the classical PtdIns 3-kinase, although the involvement of the alternative PtdIns 3-kinase cannot be excluded.

The reason for investigating the effect of CD45 co-cross-linking was primarily to manipulate intracellular levels of protein tyrosine phosphorylation. For this purpose, other studies have employed various synthetic or natural compounds interfering with tyrosine kinase activities. In some preliminary experiments we found that most commonly used inhibitors (genistein, herbimycin A) were not very effective in preventing FcγR-mediated tyrosine phosphorylation at non-cytotoxic doses. Co-cross-linking of CD45 proved to be most effective in this respect but the number of CD45 molecules expressed on the surface might be a limiting factor. This could explain the observation that FcγRIIa-mediated activation is more susceptible to co-cross-linking of CD45 when compared with FcγRIIIb-mediated activation, because FcγRIIIb expression is ten times higher than that of FcγRIIa on human neutrophils [38].

The complete inhibition of FcγR-induced enzyme release by wortmannin and the partial inhibition by CD45 co-cross-lnking (Figure 6) indicates a role for PtdIns 3-kinase in FcγR-induced degranulation. Most probably, this inhibition is due to the concomitant inhibition of the Ca^{2+} response under these conditions. Increased $[Ca^{2+}]_i$ is required to elicit a degranulation response in human neutrophils [39,40].

At this point in time, it is difficult to speculate on the exact role of PtdIns 3-kinase in early signal transduction upon FcγR cross-linking. There are some proposals in the literature for a role for the cytoskeleton in FcγR-mediated Ca^{2+} mobilization [41,42]. The enzyme PtdIns 3-kinase may be involved here, because it has been suggested that this enzyme can regulate cytoskeletal reorganization [43,44]. Recently, Rameh et al. [45] reported the ability of PtdIns(3,4,5)P_3, one of the products of PtdIns 3-kinase activity, to bind to SH2 domains, including to those of the p85 subunit of PtdIns 3-kinase itself. Our results indicate that PtdIns(3,4,5)P_3 does not play a role in the recruitment of PtdIns 3-kinase to the membrane, because this translocation proved to be insensitive to wortmannin (results not shown). However, it is conceivable that the generation of PtdIns(3,4,5)P_3 does play an instrumental role in the recruitment to the plasma membrane of phospholipase Cγ2, which is probably involved in the FcγR-induced formation of Ins(1,4,5)P_3 [46].

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