A collagen-related peptide regulates phospholipase Cγ2 via phosphatidylinositol 3-kinase in human platelets

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The collagen receptor glycoprotein VI (GPVI) induces platelet activation through a similar pathway to that used by immune receptors. In the present study we have investigated the role of phosphatidylinositol 3-kinase (PI 3-kinase) in GPVI signalling. Our results show that collagen-related peptide {CRP: [GCP*(GPP*)$_{10}$GCP*G]$_n$; P* = hydroxyproline}, which is selective to GPVI, induces formation of phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P_3] and phosphatidylinositol 3,4-bisphosphate [PI(3,4)P_2] in platelets. The increase in the two 3-phosphorylated lipids is inhibited completely by wortmannin and by LY294002, two structurally unrelated inhibitors of PI 3-kinase. The formation of inositol phosphates and phosphatidic acid (PA), two markers of phospholipase C (PLC) activation, by CRP are inhibited by between 50 and 85 % in the presence of wortmannin and LY294002. This is associated with inhibition of elevation of intracellular Ca^{2+} ($[Ca^{2+}]_i$) and aggregation. Wortmannin and LY294002 also partially inhibit elevation of Ca^{2+} by CRP in murine megakaryocytes. Microinjection of the pleckstrin-homology PH domain of Bruton's tyrosine kinase, which binds selectively to PI(3,4,5)P_3, but not the R28C ($Arg^{28} \rightarrow Cys$) mutant which binds to PI(3,4,5)P_3 with low affinity, also inhibits elevation of $[Ca^{2+}]_i$ in megakaryocytes, suggesting that it is this lipid species which mediates the action of the PI 3-kinase pathway. Studies in platelets show that the action of wortmannin and LY294002 is not mediated through an alteration in tyrosine phosphorylation of PLCγ2. These results demonstrate that PI 3-kinase is required for full activation of PLCγ2 by GPVI in platelets and megakaryocytes.

Key words: glycoprotein VI, megakaryocytes, phosphatidylinositol 3,4,5-trisphosphate.

INTRODUCTION

Platelet activation by collagen-related peptide (CRP) is mediated through a tyrosine-kinase-dependent pathway that resembles signalling by immune receptors in which pivotal roles for the Fc receptor γ-chain and the tyrosine kinase Syk have been established [1,2]. Increasing evidence suggests that activation of this pathway by CRP is mediated through the surface receptor glycoprotein IV (GPVI) and that it is independent of the integrin α2β1 [3,4]. By mimicking the triple-helical collagen structure, CRP binds to GPVI and triggers tyrosine phosphorylation of the immunoreceptor tyrosine-based activation motif (ITAM) present in the associated γ-chain via a Src family kinase [5,6]. Tyrosine phosphorylated ITAM γ-chain recruits the tyrosine kinase Syk via its two SH2 domains, leading to the activation of phospholipase Cγ2 (PLCγ2). The molecular basis of phosphorylation of PLCγ2 downstream of Syk is not established, although increasing evidence suggests a role for other proteins, including Bruton's tyrosine kinase (Btk), a Tec family protein tyrosine kinase [7,8]. PLCγ2, which is the more highly expressed PLCγ isoenzyme in platelets [9], hydrolyses phosphatidylinositol 4,5-bisphosphate [PI(4,5)P_2] to the second messengers inositol 1,4,5-trisphosphate and 1,2-diacylglycerol, which mobilize Ca^{2+} and activate PKC respectively.

PI(4,5)P_2 is also metabolised by members of the PI 3-kinase superfamily which phosphorylate inositol phospholipids at the 3 position of the inositol ring. Several types of PI 3-kinase exist in platelets from at least two different classes [10]. The class I heterodimeric p85/p110 is highly expressed in human platelets [11]. The 110 kDa subunit possesses both a lipid kinase and a serine/threonine kinase activity. PI(4,5)P_2 is the preferred substrate for members of the class I family. Members of the class II family have a C2 domain which serves as a Ca^{2+}-sensitive phospholipid-binding site [11]. These PI 3-kinases phosphorylate PI preferentially relative to phosphatidylinositol 4-monophosphate [PI(4)P] and PI(4,5)P_2.

Much of the attention on this superfamily has focused on the formation of the second messenger phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P_3], which regulates a wide variety of cellular responses through translocation of proteins to the membrane, usually through interaction with their pleckstrin-homology (PH) domains, e.g. Tec family protein tyrosine kinases [12–14]. The other well-characterized 3-phosphorylated inositol phospholipid is PI(3,4)P_2, which also appears to be a second

Abbreviations used: Btk, Bruton's tyrosine kinase; $[Ca^{2+}]_i$, concentration of intracellular calcium ions; CRP, collagen-related peptide; DAG, 1,2-diacylglycerol; GPVI, glycoprotein VI; GST, glutathione S-transferase; ITAM, immunoreceptor tyrosine-based activation motif; mAb, monoclonal antibody; NP-40, Nonidet P40; PA, phosphatidic acid; PH, pleckstrin homology; PI 3-kinase, phosphatidylinositol 3-kinase; PI(4)P phosphatidylinositol 4-monophosphate; PI(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PI(3,4)P_2, phosphatidylinositol 3,4-bisphosphate; PI(3,4,5)P_3, phosphatidylinositol 3,4,5-trisphosphate; PLC, phospholipase C; SHIP, Src homology 2 domain containing inositol 5-phosphatase; ACD, acid citrate/dextrose.

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messenger. PI(3,4)P_2 is formed by dephosphorylation of PI-(3,4,5)P_3 by, for example, the recently described Src homology 2 domain containing inositol 5-phosphatase (SHIP) [15], or by the action of PI 3-kinase on PI(4)P or PI 4-kinase on PI(3)P [10].

PI 3-kinase is implicated in the regulation of PLCγ isoforms downstream of growth-factor and immune receptors [16]. Falasca et al. demonstrated that the N-terminal PH domain of PLCγ1 binds to PI(3,4,5)P_3 and migrates to the membrane through a PI 3-kinase sensitive pathway as revealed by coupling to green fluorescent protein [17]. PI(3,4,5)P_3 also increases the activity of PLCγ1 when incorporated into lipid micelles, an action mediated by the tandem Src homology 2 domains in the phospholipase [18,19]. The functional relevance of these interactions is indicated by the observation that wortmannin decreases platelet-derived-growth-factor activation of PLCγ1 in fibroblasts [17,18]. PI 3-kinase activation is also required for phosphorylation, activation and translocation of PLCγ1 following stimulation of FcϵRI receptor in a mast-cell line [20]. However, in the same cells, the regulation of PLCγ2 seems to be independent of PI 3-kinase [20].

In the present study we have investigated the role of PI 3-kinase in GPVI receptor signalling in human platelets stimulated by CRP. Our results provide evidence for a major role for PI 3-kinase in the activation of PLCγ2 via GPVI and suggest that PI(3,4,5)P_3 is the major PI 3-kinase product underlying this event.

MATERIALS AND METHODS

Materials

CRP {[GCP*(GPP*)$_{10}$GCP*G]$_n$, P* = hydroxyproline; the peptide is cross-linked through the C-terminal cysteines} was kindly given by Dr. M. J. Barnes and Dr. R. W. Farndale (Department of Biochemistry, University of Cambridge, Cambridge, U.K.) [21]. Tween-20, Protein A-Sepharose CL-4B, PMSF, protease inhibitors, thrombin, apyrase and poly-L-lysine (molecular mass 70 000) and prostacyclin were from Sigma (Poole, Dorset, U.K). HPLC Column SAX partisphere was from Whatman (Maidstone, Kent, U.K.). The anti-phosphotyrosine monoclonal antibody (mAb) 4G10 was from Upstate Biotechnology Inc. (TCS Biologicals Ltd., Botolph Claydon, Bucks., U.K.). Anti-PLCγ1 (530) and anti-PLCγ2 (Q-20) polyclonal antibody were from Santa Cruz (supplied by Autogen Bioclear, Calne, Wilts., U.K.). LY294002 was purchased from Calbiochem–Novabiochem (Nottingham, U.K.). Nonidet P40 (NP40) was from BDH (Poole, Dorset, U.K.). [^{32}P]P_i (3000 Ci/mmol), *myo*-[^{3}H]inositol (18.2 Ci/mmol) and secondary antibody and ECL® (enhanced chemiluminescence) reagents were from Amersham International (Cardiff, Wales, U.K.). Fura-2 acetoxymethyl ester was from Molecular Probes (Eugene, OR, U.S.A). PVDF membrane was purchased from Bio-Rad (Hemel Hempstead, Herts., U.K.). Fura-2 acetoxymethyl ester, wortmannin and LY294002 were dissolved in DMSO. Other reagents were made up in saline. The concentration of DMSO in the incubation never exceeded 1:1000 final dilution, and an equivalent volume of DMSO was always present in control samples.

Preparation of human platelets and stimulation

Human blood was taken from drug-free volunteers on the day of the experiment using acid/citrate/dextrose (ACD; 120 mM sodium citrate/110 mM glucose/80 mM citric acid) as anticoagulant. Platelet-rich plasma was obtained by centrifugation at 200 ***g*** for 20 min. Platelets were isolated by centrifugation at 1000 ***g*** for 10 min in the presence of prostacyclin (0.1 μg/ml). Then platelets were resuspended in 25 ml of a modified Tyrode's/Hepes buffer (134 mM NaCl/0.34 mM Na_2HPO_4/2.9 mM KCl/12 mM $NaHCO_3$/20 mM Hepes/5 mM glucose/1 mM $MgCl_2$, pH 7.3) and 3 ml of ACD in the presence of prostacyclin (0.1 μg/ml). Platelets were centrifuged at 1000 ***g*** for 10 min and resuspended at a concentration of 5×10^8 cells/ml in Tyrode's/Hepes buffer containing EGTA (1 mM) and indomethacin (10 μM). EGTA was absent in aggregation studies. Stimulation was performed in an aggregometer at 37 °C under continuous stirring (1200 rev./min).

Immunoprecipitation studies

Samples were stopped by adding an equal volume of ice-cold lysis buffer [20 mM Tris/300 mM NaCl/2 mM EGTA/2 mM EDTA/2 % (v/v) NP40/1 mM PMSF/2 mM Na_3VO_4/10 μg/ml leupeptin/10 μg/ml aprotinin/1 μg/ml pepstatin A, pH 7.3]. Non-lysed cells and debris were removed by centrifugation and lysates were precleared by mixing with Protein A–Sepharose for 1 h at 4 °C [20 μl of a 50 % (w/v) suspension of Protein A–Sepharose in TBS-T (20 mM Tris/137 mM NaCl/0.1 % Tween 20, pH 7.3)]. PLCγ2 were immunoprecipitated from 1 ml of lysate using 1 μg/ml antibody. Following rotation for 1 h, Protein A–Sepharose was added to each sample and rotated for a further 1 h. The pellet of Protein A–Sepharose was washed in lysis buffer and three times in TBS-T before the addition of Laemmli buffer [4 % (w/v) SDS/10 % (v/v) mercaptoethanol/20 % (v/v) glycerol/50 mM Tris, pH 6.8] and boiling for 10 min.

Immunoblotting studies

Reactions were stopped by adding an equal volume of Laemmli buffer, and samples were boiled for 10 min. Proteins were separated by SDS/PAGE and transferred to PVDF membranes which were blocked by incubation in 10 % (w/v) BSA dissolved in TBS-T. Primary and secondary antibodies were diluted in TBS-T containing 2 % (w/v) BSA and incubated with membranes for 1 h at room temperature. Blots were washed for 2 h in TBS-T following each incubation and then developed using the ECL® detection system. Anti-phosphotyrosine and anti-PLCγ2 were used at 1 μg/ml and 1:100 respectively. Horseradish-peroxidase-conjugated secondary antibody was diluted 1:10 000.

Platelet labelling and phosphoinositide analysis

Platelets suspended in phosphate-free Hepes buffer were incubated with 0.6 mCi/ml of [^{32}P]P_i for 1 h at 37 °C [22]. After one wash, platelets were resuspended at 1×10^9/ml in Tyrode's buffer plus 1 mM Ca^{2+} and 10 μM indomethacin and left for 15 min. Experiments were performed in an aggregometer at 37 °C and stirring at 1200 rev./min. Reactions were stopped by addition of chloroform/methanol (1:1, v/v). After phospholipid extraction, deacylated lipids were resolved by HPLC (strong-anion-exchange column partisphere SAX) and eluted by a linear gradient [$(NH_4)_2HPO_4$, 0–1 M] as described in [22,23]. Radiolabelled phosphoinositides were identified by comparison with standards. For PA formation, extracted lipids were resolved by TLC and directly quantified by PhosphorImager analysis using a 445SI machine (Molecular Dynamics) [23].

Measurement of Ca^{2+} and [^{3}H]inositol phosphates

Washed platelets incubated with Fura-2 acetoxymethyl ester (2 μM, 45 min, 37 °C). After being washed, platelets were resuspended at 5×10^8 platelets/ml. Stimulation was performed at 10^8/ml in a Perkin–Elmer LS50B spectrofluorimeter at 37 °C

with agitation in the presence of 0.1 mM EGTA. $[Ca^{2+}]_i$ was calculated using the software FLWinlab (Perkin–Elmer).

For inositol phosphates platelets were labelled with 50 μCi/ml of *myo*-[^{3}H]inositol for 3 h at 37 °C, washed and resuspended at a concentration of 4×10^8cells/ml as described [23]. LiCl (10mM) was added to each sample to inhibit conversion of inositol phosphates into free inositol. Reactions were stopped after 5 min of stimulation with 0.94 ml of chloroform/methanol/HCl (50:100:1, by vol.). Water (0.31 ml) and chloroform (0.31 ml) were then added. After separation of phases, total [^{3}H]inositol phosphates (mono-, bis- and tris-phosphates) were eluted by Dowex anion-exchange chromatography as described in [24].

Isolation of mouse megakaryocytes and single-cell studies

Mouse megakaryocytes were prepared as previously described and plated on to poly-L-lysine-coated coverslips [5]. Excess suspension was removed and replaced by Hanks buffer. All experiments were carried out with an extracellular calcium-ion concentration ('$[Ca^{2+}]_e$') of 200 μM at room temperature (22 ± 2 °C). Cells were viewed in an inverted microscope (Axiovert 35). Stage III/IV megakaryocytes, identified as previously described [5], were identified on the basis of size and morphology. Microinjection of Fura-2 and recombinant protein was carried out using an Eppendorf micromanipulator 5170 and microinjector 5242. Fura-2 pentapotassium salt was dissolved in standard intracellular buffer (125 mM potassium glutamate/1 mM $MgCl_2$/50 μM EGTA/5 mM Hepes, pH 7.2) and was present in the injection needle at a concentration of 2.5 mM, resulting in an estimated intracellular concentration of $\leqslant$ 200 μM. cDNA encoding the PH domain of Btk (corresponding to amino acids 2–174) was amplified by PCR from mouse Btk cDNA cloned previously [25]. The R28C ($Arg^{28} \rightarrow Cys$) mutation was generated using a two-step PCR method as described in [25]. After checking protein purity by SDS/PAGE, the glutathione S-transferase (GST) recombinant fusion proteins are present in the injection needle at different range of concentration (2.7, 0.27 and 0.027 μM), giving estimated intracellular concentrations of $\leqslant$ 270, 27 and 2.7 nM respectively. Single-cell digital imaging was carried out using Ionvision software (Improvision, Warwick, U.K.). Fluorescence video images were captured at excitation wavelengths of 340 nm and 380 nm, with emission at 510 nm. Calculation of $[Ca^{2+}]_i$ from the 340/380 ratio was performed using a calibration curve made with standard solutions of various free Ca^{2+} concentrations, applying a viscosity correction factor. Data are presented as both the peak value of $[Ca^{2+}]_i$ after agonist addition and the rise in $[Ca^{2+}]_i$ above the basal level, taken immediately prior to agonist addition.

For immunostaining, megakaryocytes were isolated as described above. Megakaryocytes were activated by CRP (4 μg/ml) for 90 s (or not activated) and then fixed with 3.7% (w/v) formaldehyde for 10 min. Megakaryocytes were permeabilized with Triton X-100 (0.2%) for 10 min. Unspecific sites were saturated with 2% BSA and megakaryocytes were immunostained for PLCγ2 or PLCγ1. Primary antibody was detected by FITC-conjugated donkey anti-rabbit IgG. Megakaryocytes were viewed in an inverted microscope (Axiovert S 100) and analysed by deconvolution using Openlab software (Improvision, Warwick, U.K.).

Analysis of data

Each experiment was performed at least three times. Results are shown as means $\pm$ S.E.M. Differences were analysed by an unpaired Student's *t* test. In each case, $P < 0.05$ was taken as the minimum value to indicate statistical significance.

RESULTS

CRP stimulates formation of P(3,4,5)P_3 and P(3,4)P_2 in human platelets

To investigate if GPVI receptor signalling is coupled to the PI 3-kinase pathway, we measured the formation of 3-phosphorylated phosphoinositides by HPLC. CRP stimulated a five-fold increase in PI(3,4,5)P_3 at 1 min, which did not increase further by 2 min.

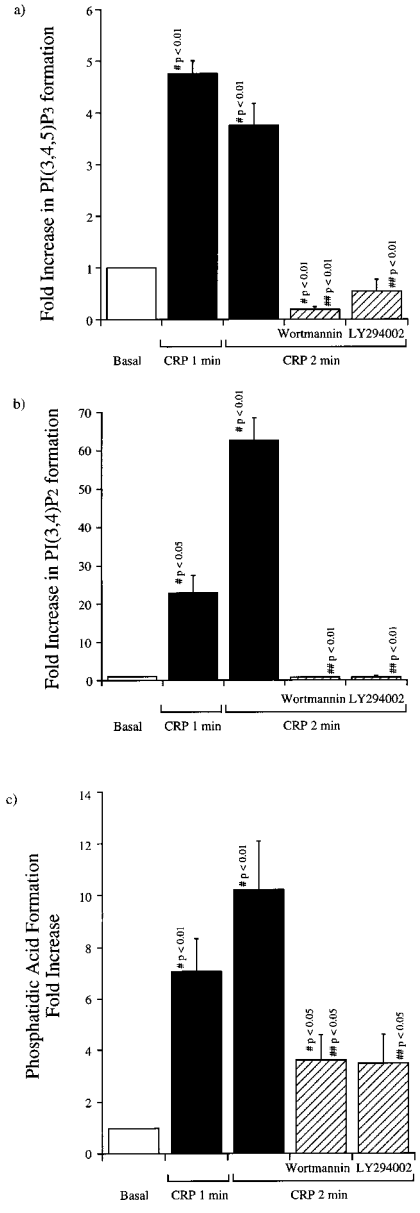

Figure 1 CRP stimulates formation of PI(3,4,5)P_3, PI(3,4)P_2 and PA in human platelets

Washed platelets (1×10^9/ml), labelled with [^{32}P]P_i, were preincubated with vehicle alone (DMSO), wortmannin (100 nM) or LY294002 (50 μM) for 15 min and then stimulated with 5 μg/ml CRP in an aggregometer cuvette with stirring at 37 °C. The reaction was stopped by addition of 4 vol. of chloroform/methanol (1:1, v/v). Phospholipids were separated and analysed as described in the Materials and methods section. (**a**) PI(3,4,5)P_3 formation; (**b**) PI(3,4)P_2 formation; (**c**) PA formation. Results are fold increases (means $\pm$ S.E.M.) from three experiments. The *P* value corresponds to results significantly different from basal (#) or following stimulation with CRP for 2 min (##).

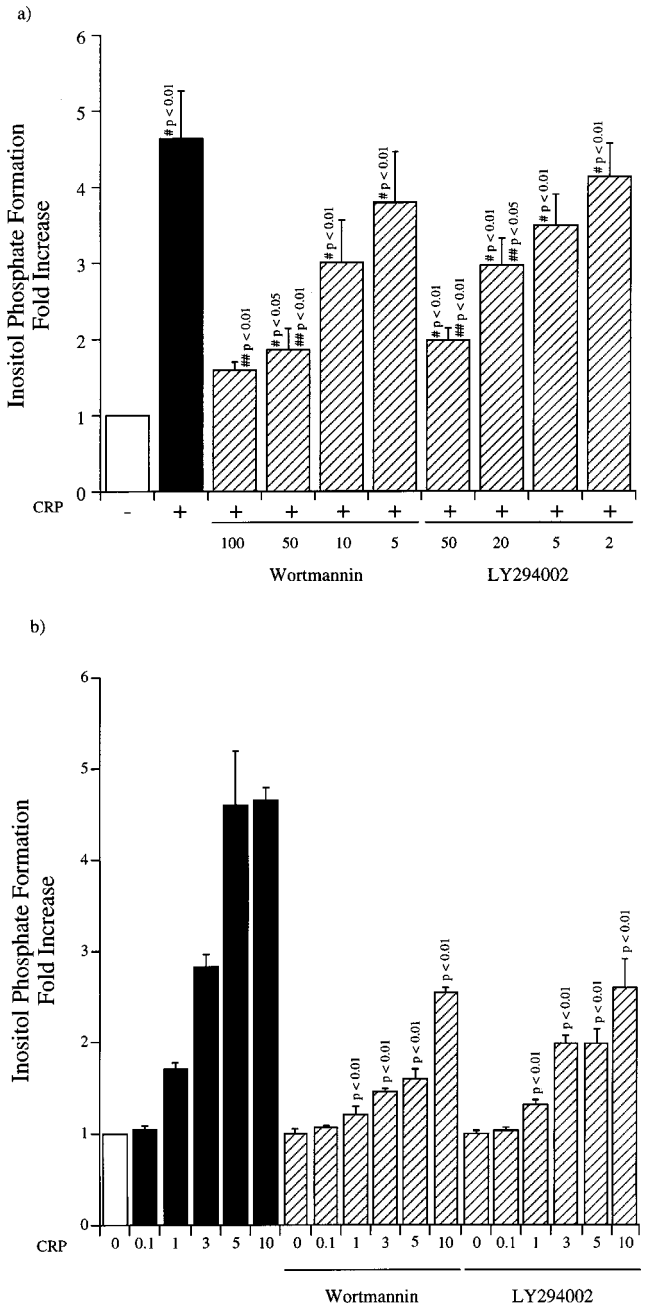

Figure 2 CRP stimulates formation of inositol phosphates in human platelets

Washed platelets (1×10^9/ml), labelled with *myo*-[^{3}H]inositol, were preincubated with vehicle alone (DMSO) or the indicated concentrations of wortmannin or LY294002 for 15 min and then stimulated with CRP for 5 min in an aggregometer cuvette with stirring at 37 °C. Inositol phosphates were separated from *myo*-[^{3}H]inositol on Dowex anion-exchange columns. (**a**) Concentration–response curve to wortmannin and LY294002 against CRP (5 μg/ml) and (**b**) to CRP in the presence of wortmannin (100 nM) or LY294002 (50 μM). Results are expressed as fold increases (mean ± S.E.M.) for (**a**) five or (**b**) three experiments. The *P* value corresponds to results significantly different from basal (#) or following stimulation with CRP for 2 min (##) or from the respective control (Figure 2b).

Preincubation with 100 nM wortmannin for 15 min at 37 °C before stimulation by CRP completely inhibited the rise in $PI(3,4,5)P_3$ (Figure 1a). LY294002, which is a structurally unrelated PI 3-kinase inhibitor, also fully inhibited the increase of $PI(3,4,5)P_3$ in response to CRP (Figure 1a).

In contrast, the peak level of $PI(3,4)P_2$ was delayed in comparison with $PI(3,4,5)P_3$. A 28-fold increase over basal was found at 1 min in response to CRP, which increased to 65-fold by 2 min. This is consistent with $PI(3,4)P_2$ being a breakdown product of $PI(3,4,5)P_3$. In support of this, formation of $PI(3,4,5)P_3$ by CRP was potentiated in mice platelets deficient in the 5-phosphatase SHIP, whereas formation of $PI(3,4)P_2$ was reduced (J.-M. Pasquet, V. Duronio, G. Krystal and S. P. Watson, unpublished work). Wortmannin (100 nM) and LY294002 (50 μM) completely inhibited formation of $PI(3,4)P_2$ (Figure 1b).

Formation of inositol phosphates and PA by CRP is dependent on the PI 3-kinase pathway

To follow the degree of activation of PLCγ2 by CRP, we measured PA, the metabolite of 1,2-diacylglycerol (DAG), and inositol phosphates in platelets labelled with [^{32}P]P_i and [^{3}H]inositol respectively. The latter studies were carried out in the presence of LiCl to prevent conversion into inositol. CRP (5 μg/ml) stimulated a 7-fold increase in PA over basal at 1 min, which increased to 10-fold at 2 min (Figure 1c). In comparison, CRP (5 μg/ml) stimulated a 4.5-fold increase in inositol phosphates after 5 min over basal (Figure 2a).

Preincubation with 100 nM wortmannin or 50 μM LY294002 significantly ($P < 0.01$) decreased the formation of PA in response to CRP by approx. 75 % in each case (Figure 1c). The two inhibitors also reduced the formation of total inositol phosphates in response to CRP with a concentration–response relationship similar to that previously reported for inhibition of PI 3-kinase (Figure 2a). Concentrations of wortmannin and LY294002 which completely inhibit formation of 3-phosphorylated lipids (see Figures 1a and 1b) decreased the response to CRP (5 μg/ml) by approx. 70 %. The effect of the two PI 3-kinase inhibitors on the concentration–response curve for formation of inositol phosphates by CRP was also investigated (Figure 2b). Wortmannin and LY294002 inhibited the response to CRP by between 50 and 70 % throughout the length of the concentration–response curve, but the response was not completely inhibited. These results demonstrate that the activity of PLCγ2 is heavily dependent on PI 3-kinase, but that a minor component is independent of this pathway. In contrast, PI 3-kinase inhibitors had no significant effect on inositol phosphate formation induced by thrombin (results not shown).

Role of PI 3-kinase in Ca^{2+} mobilization and aggregation in platelets

CRP stimulated a rapid, concentration-dependent increase in intracellular Ca^{2+} in Fura-2-loaded platelets to an initial peak followed by a sustained plateau. Preincubation of platelets with 100 nM wortmannin partially decreased both components of the increase in Ca^{2+}, although the profile of the response was not changed (Figure 3a). In contrast, wortmannin (100 nM) had no significant effect on Ca^{2+} mobilization by thrombin (results not shown). LY294002 (50 μM) was observed to have a small (< 20 %) inhibitory effect on Ca^{2+} mobilization by thrombin, and so its action against CRP was not analysed.

CRP induced platelet aggregation in a concentration-dependent manner with a maximal response observed at 3–5 μg/ml. The two PI 3-kinase inhibitors fully blocked aggregation to low

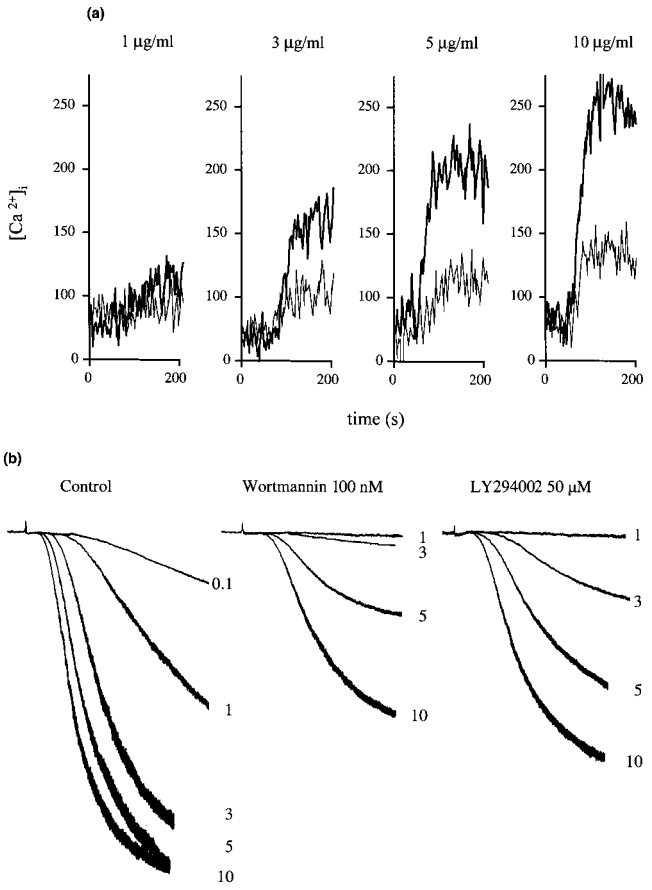

Figure 3 Ca^{2+} mobilization and aggregation induced by CRP is inhibited by PI 3-kinase inhibitors in human platelets

(**a**) Fura-2-loaded (1×10^8/ml) control (**bold** trace) or wortmannin treated-platelets (thin trace) were stimulated by the indicated CRP concentration in a spectrofluorimeter cuvette with a dual excitation at 340/380 nm and an emission recorded at 510 nm. Wortmannin (100 nM) was preincubated as in Figure 1. $[Ca^{2+}]_i$ was calculated as described in the Materials and methods section. (**b**) Washed platelets (5×10^8/ml) preincubated with DMSO (control), wortmannin (100 nM) or LY294002 (50 μM) for 15 min, were stimulated by the indicated CRP concentration in an aggregometer cuvette with stirring at 37 °C. Results are from one experiment representative of six.

concentrations of CRP (0.1–1 μg/ml), whereas partial recovery was seen at higher concentrations of the collagen peptide (Figure 3b). For a concentration of 5 μg/ml CRP, aggregation was inhibited by $86.4 \pm 6.1\,\%$ ($n = 5$, $P < 0.01$) in the presence of wortmannin and by $75.3 \pm 9.8\,\%$ ($n = 5$, $P < 0.01$) in the presence of LY294002 (all measurements were made after a period of 4 min of stimulation when aggregation reach a plateau). It was noticeable that a concentration of CRP (1 μg/ml) which gave submaximal aggregation in control platelets stimulated a comparable level of aggregation and inositol phosphates to that induced by a fivefold greater concentration of CRP in the presence of wortmannin and LY294002 (Figures 2b and 3b), suggesting a causal relationship.

PI 3-kinase inhibitors do not prevent PLCγ2 phosphorylation

The two PI 3-kinase inhibitors had no apparent effect on whole-platelet tyrosine phosphorylation induced by CRP (results not shown). In order to investigate whether they had an effect on PLCγ2 phosphorylation, the phospholipase was immunoprecipitated using a specific antiserum. CRP (5 μg/ml) induced strong tyrosine phosphorylation of PLCγ2, and this was not altered significantly in the presence of wortmannin (20–100 nM; Figure 4a) or LY294002 (2–50 μM, Figure 4b). These results demonstrate that PLCγ2 phosphorylation is independent on PI 3-kinase activity.

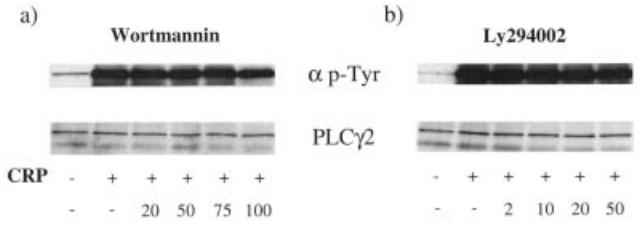

Figure 4 PI 3-kinase inhibitors do not inhibit tyrosine phosphorylation of phospholipase Cγ2 by CRP in human platelets

Washed platelets (5×10^8/ml) were preincubated with (**a**) wortmannin or (**b**) LY294002 for 15 min and stimulated with 5 μg/ml CRP in an aggregometer cuvette with stirring at 37 °C for 2 min. The reaction was stopped by addition of ice-cold lysis buffer. After immunoprecipitation for PLCγ2, samples were subjected to SDS/PAGE and transferred to PVDF membrane. Immunoblotting was performed as described in the Materials and methods section with the mAb 4G10 anti-phosphotyrosine (upper panel) or with the antibody Q-20 anti-PLCγ2 (lower panel). Results are representative of three experiments.

PI(3,4,5)P_3 is required for Ca^{2+} mobilization in CRP-stimulated mouse megakaryocytes

We investigated the role of PI 3-kinase in Ca^{2+} mobilization by CRP in the platelet precursor cell, the megakaryocyte, using single-cell video imaging. Results are shown as example of records from single cells (Figure 5a) and as means $\pm$ S.E.M. (Figure 5b). CRP (4 μg/ml) stimulates a rapid rise in Ca^{2+} which slowly returns to basal. The peak increase in Ca^{2+} was 239 ± 46 nM ($n = 9$) above basal. In the presence of 100 nM wortmannin, the peak response to CRP was decreased to 60 ± 15 nM ($P < 0.01$; $n = 9$), with a similar time course to that in control cells. Wortmannin had no significant effect on the response to thrombin.

To identify the PI 3-kinase product, which is involved in this pathway, we investigated the effect of microinjection of the wild-type PH domain of Btk on Ca^{2+} mobilization, which binds selectively to PI(3,4,5)P_3 relative to PI(3,4)P_2 and PI(4,5)P_2 [26,27]. The selectivity of the wild-type Btk PH domain enables it to be used to prevent the action of PI(3,4,5)P_3 through chelation. The R28C mutant of the Btk PH domain, which binds to PI(3,4,5)P_3 with a lower affinity and also with a similar affinity to PI(3,4)P_2 and PI(4,5)P_2, served as a control.

The wild-type Btk PH domain was introduced into murine megakaryocytes by microinjection at an estimated intracellular concentration of 27 nM, which is close to the K_d for inositol 1,3,4,5-tetrakisphosphate and PI(3,4,5)P_3 [28,29]. This domain inhibited the peak of Ca^{2+} to CRP by greater than 70 %, but had no significant effect on the response to thrombin (Figure 5b). The small increase in Ca^{2+} induced by CRP in the presence of the wild-type Btk PH domain was maintained for several minutes. In contrast, the mutant Btk PH domain (R28C) had no effect on Ca^{2+} mobilization induced by CRP when microinjected at the same concentration (Figure 5a, trace iv). A tenfold lower concentration of the wild-type Btk PH domain had no significant effect on the Ca^{2+} response induced by CRP, whereas a tenfold higher concentration of both the wild-type and the mutant Btk

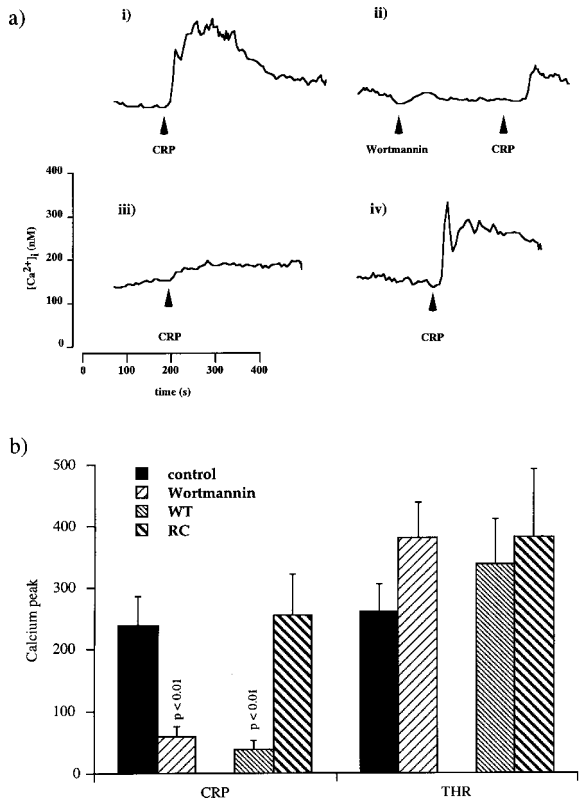

Figure 5 Effect of wortmannin and the PH domain of Btk on Ca^{2+} mobilization by CRP using mouse megakaryocytes

(**a**) Megakaryocytes from wild-type Balb/c mice were isolated from bone marrow as described in the Materials and methods section. Stage III/IV megakaryocytes were injected with Fura-2 (traces i and ii), Fura-2 and 27 nM GST–PH domain of the wild-type Btk (trace iii) or Fura-2 and 27 nM GST–PH (R28C) domain of the mutant Btk (trace iv). Wortmannin (100 nM) was given 5 min before CRP (trace ii). Cells were stimulated with 4 μg/ml CRP. Fura-2 fluorescence was measured and $[Ca^{2+}]_i$ determined as described in the Materials and methods section. Each trace is representative of those from between 7 and 11 cells taken from at least four different mice. (**b**) The histogram shows the peaks in $[Ca^{2+}]_i$ over basal in response to 4 μg/ml CRP and 1 unit/ml thrombin. Results are expressed as means $\pm$ S.E.M.; the *P* value was calculated in comparison with the respective control (CRP or thrombin-stimulated) and indicated only when *P* is < 0.01.

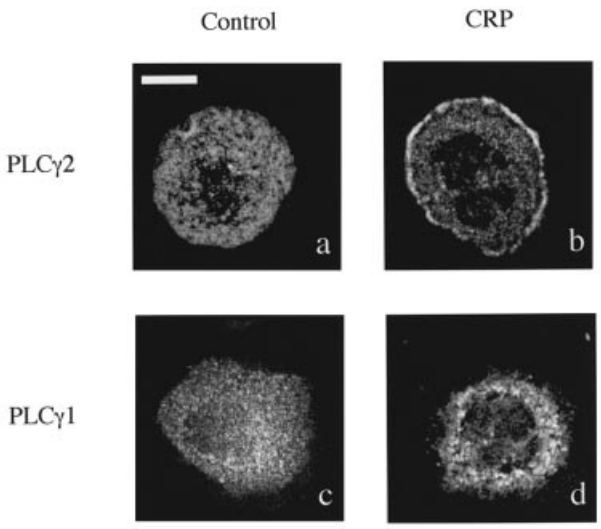

Figure 6 CRP stimulates the translocation of phospholipase Cγ2, but not phospholipase Cγ1, in murine megakaryocytes

The micrographs shown mouse megakaryocytes control (**a**, **c**), or activated by 4 μg/ml CRP for 90 s (**b**, **d**), immunostained for PLCγ2 (**a**, **b**) or PLCγ1 (**c**, **d**). Immunostained cells were revealed by fluorescence microscopy. Immunofluorescence images were analysed by deconvolution as described in the Materials and methods section. The scale bar in the micrograph represents 5 μm. Each image is representative of ten megakaryocytes obtained in a minimum of three experiments on different animals.

PH domains decreased the Ca^{2+} response triggered by CRP (results not shown).

CRP stimulates translocation of phospholipase Cγ2, but not phospholipase Cγ1, in mouse megakaryocytes

Platelets (and therefore also megakaryocytes) express both PLCγ1 and PLCγ2 isoforms, but only PLCγ2 undergoes tyrosine phosphorylation in response to CRP, a result that has given rise to the belief that this isoform underlies platelet activation [24]. The demonstration in the present study, however, for a role of the PI 3-kinase pathway in the regulation of PLCγ activity raises the possibility that activation may also occur independently of tyrosine phosphorylation for both PLCγ1 and PLCγ2 isoforms. This was tested by monitoring the location of PLCγ1 and PLCγ2 in murine megakaryocytes following activation by CRP. CRP stimulated marked translocation of PLCγ2 to the surface membrane, whereas PLCγ1 remained in the cytosol (Figure 6).

DISCUSSION

Our results show that two inhibitors of PI 3-kinase, wortmannin and LY294002, completely inhibit CRP-induced formation of $PI(3,4)P_2$ and $PI(3,4,5)P_3$ in platelets, and partially block formation of inositol phosphates and PA. Consistent with this, both inhibitors partially block Ca^{2+} elevation and aggregation by CRP in platelets, and Ca^{2+} elevation in the megakaryocyte. The PH domain of Btk, which binds with a high affinity to $PI(3,4,5)P_3$, also partially inhibits elevation of Ca^{2+} in megakaryocytes. These results demonstrate that PI 3-kinase is required for maximal activation of PLCγ activity by CRP, but that a component of PLC activity is regulated independently of this pathway.

There are several possible mechanisms whereby elevation of $PI(3,4,5)P_3$ could regulate the activity of PLCγ2 by CRP. These include the possible interaction of $PI(3,4,5)P_3$ with the N-terminal PH domain or tandem SH2 domains of PLCγ2, as reported for PLCγ1 [18–20]. Gratacap et al. have recently shown that PLCγ2 is associated with $PI(3,4,5)P_3$, consistent with a direct role for $PI(3,4,5)P_3$ in the regulation of PLCγ2 [23]. They also showed that exogenous $PI(3,4,5)P_3$ restores the activity of PLCγ2 in wortmannin-treated permeabilized platelets stimulated by the low-affinity immune receptor FcγRIIA [23], which signals through a pathway similar to that used by GPVI. This observation suggests that $PI(3,4,5)P_3$ is the active product, consistent with the inhibitory effect of the wild-type Btk PH domain which binds to $PI(3,4,5)P_3$ [27].

The role of the PI 3-kinase pathway in the regulation of PLCγ2 could also be mediated by an indirect action. The wild-type PH domain of Btk binds with a high affinity to $PI(3,4,5)P_3$ [26,27], and recent evidence suggests that this support its association with the membrane [29]. There is strong evidence for a role of Btk in PLCγ2 phosphorylation. For example, phos-

phorylation of PLCγ2 by the B-cell receptor is inhibited in chick DT40 cells engineered to lack Btk [8]. Scharenberg et al. have shown that Btk and other members of the Tec family protein tyrosine kinases phosphorylate PLCγ2 *in vitro* [14]. It is also shown that tyrosine phosphorylation of PLCg2 in response to collagen is inhibited in platelets from patients with X-linked agammaglobulinaemia caused by presence of a mutant form of Btk [7]. However, the observation that neither of the PI 3-kinase inhibitors had a significant effect on the tyrosine phosphorylation of PLCγ2 argues against an indirect action mediated through inhibition of Btk-mediated phosphorylation of PLCγ2. Alternatively, Scharenberg et al. have proposed that the major role of the interaction of PI(3,4,5)P_3 with Btk is to enable the optimal orientation of the substrate lipid, PI(4,5)P_2, to PLCγ2 in the membrane, and this pathway would be inhibited by wortmannin and LY294002 [14].

The lack of full inhibition of activation of PLCγ2 by wortmannin and LY294002 demonstrates that PI 3-kinase inhibition can be by-passed. This second pathway may act either independently of PI 3-kinase or in synergy. We speculate that the PI 3-kinase-independent pathway involves recruitment of PLCγ2 to the membrane through interaction with the adapter proteins Lat or SLP76, which fulfill this role in T-cells [30,31] and platelets activated by GPVI ([32]; and J.-M. Pasquet, B. Gross, L. Quek, N. Asazuma, W. Zhang, C L. Sommers, E. Schweighoffer, V. Tybulewicz, B. Judd, J. R. Lee, G. Koretzky, P. E. Love, L. E. Samelson and S. P. Watson, unpublished work). PLCγ2 may be recruited to the membrane by association with the adapter proteins, while formation of PI(3,4,5)P_3 is required to maintain PLCγ2 activity, possibly by maintaining association to the membrane.

In summary, PI 3-kinase activity is required for full activation of PLCγ2 by CRP, with PI(3,4,5)P_3 being the PI 3-kinase product underlying this event. These observations provide further evidence that signalling by CRP through GPVI shares many features with signalling by the immune receptor family.

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