Thrombin stimulates wortmannin-inhibitable phosphoinositide 3-kinase and membrane blebbing in CHRF-288 cells

Geeta S. VEMURI, Jin ZHANG, Rusong HUANG, James H. KEEN and Susan E. RITTENHOUSE*

Department of Pharmacology/Jefferson Cancer Institute and Cardeza Foundation for Hematological Research, Thomas Jefferson University, Philadelphia PA 19107, U.S.A.

We have investigated thrombin-stimulated morphological changes and the activation of phosphoinositide 3-kinase (PI 3-K), as manifested by the accumulation of $\text{PtdIns}(3,4)\text{P}_2$ and $\text{PtdIns}(3,4,5)\text{P}_3$ (labelled with ^{32}P or myo - ^3H]inositol), in CHRF-288 cells, a leukaemic cell line derived from a platelet progenitor cell. We report that these cells, when exposed to thrombin or SFLLRN (the peptide Ser-Phe-Leu-Leu-Arg-Asn, a thrombin-receptor ligand) rapidly change shape, forming membrane ‘blebs’, detectable by differential interference contrast or confocal microscopy, as well as labelled 3-phosphorylated phosphoinositides. The ‘blebs’ are distinguishable from ‘ruffles’ or lamellae, since they do not contain phalloidin-detectable actin. Studies with permeabilized cells indicate that PI 3-K is activated synergistically by thrombin + guanosine 5'-[γ -thio]triphosphate.

Two forms of PI 3-K, i.e. PI 3-K(γ) and p85/PI 3-K, regulated by $G\beta\gamma$ subunits of heterotrimeric G-protein and the small G-protein Rho, respectively, are present in these cells, as is true for platelets. Wortmannin, a known potent and specific inhibitor of PI 3-K activities, inhibits thrombin-stimulated accumulation of 3-phosphorylated phosphoinositides in a dose-dependent manner ($\text{IC}_{50} \sim 10\text{ nM}$), without affecting phospholipase C activation. Pretreatment of CHRF-288 cells with either wortmannin (100 nM) or an unrelated synthetic PI 3-K inhibitor, LY294002 (50 μM), abolishes thrombin-receptor-stimulated blebbing. These results suggest that thrombin-stimulated accumulation of 3-phosphorylated phosphoinositide(s) is required for the shape-change response in CHRF-288 cells.

INTRODUCTION

α -Thrombin, a serine protease, possesses hormonal and growth-factor activities and also promotes a diverse range of biological functions which are essential for haemostasis and wound healing. Activation of the thrombin receptor induces growth of vascular smooth-muscle cells, shape changes in neural cells and activation of platelets, leading to aggregation and secretion as well as the formation of microvesicles. A thrombin receptor has been characterized and was found to contain seven transmembrane domains and to be activated by the resulting N-terminal region of the newly hydrolysed receptor (the peptide Ser-Phe-Leu-Leu-Arg-Asn, SFLLRN [1]). Such receptors are usually coupled to heterotrimeric GTP-binding proteins.

Early studies from our laboratory have shown that thrombin causes rapid accumulation of two 3-phosphorylated phosphoinositides (3-PPI), i.e. $\text{PtdIns}(3,4,5)\text{P}_3$ and $\text{PtdIns}(3,4)\text{P}_2$. The kinetics of their accumulation are consistent with their role in cytoskeletal reorganization/aggregation in platelets [2]. Further studies indicated the association of activated phosphoinositide 3-kinase (PI 3-K) with a focal adhesion-like integrin ($\alpha_{\text{IIb}}\beta_3$)/vinculin/talin-containing membrane skeleton complex in such platelets [3]. The accumulation of thrombin-stimulated 3-PPI in platelets is regulated by both the small GTP-binding protein Rho and $G\beta\gamma$, the $\beta\gamma$ subunit of heterotrimeric GTP-binding protein [4], affecting respectively the PI 3-Ks designated ‘p85/PI 3-K’ and ‘PI 3-K(γ)’.

We have examined the activation of phospholipase C (PLC) and PI 3-K by serum or lysophosphatidic acid (LPA) added to

CHRF-288 cells, a leukaemic cell line derived from a platelet progenitor cell, the megakaryoblast [5]. We correlated these events with the relative growth-promoting activities of these two agonists by utilizing wortmannin, a potent and specific inhibitor of both forms of PI 3-K [6–8], and concluded that PI 3-K activity is necessary, but not sufficient, for a proliferative response. We now report on the effects of thrombin or thrombin-receptor-derived peptide in promoting the activation of PLC and PI 3-K in CHRF-288 cells, morphological changes, and proliferation, and the modification by wortmannin or an unrelated PI 3-K inhibitor, LY294002 [9], of such responses. In addition, we describe the PI 3-K species present in these cells, in comparison with platelets.

MATERIALS AND METHODS

Materials

^{32}P P_i (8500–9120 Ci/mmol) was purchased from ICN, and myo -[2- ^3H (N)]inositol was from Du Pont–New England Nuclear. Dulbecco’s Modified Eagle Medium (DMEM) and horse serum were obtained from GIBCO–BRL Life Technologies. α -Thrombin was purchased from Hemetec (Burlington, VT, U.S.A.). SFLLRN was synthesized by the Jefferson Cancer Institute’s peptide facility. Chromatography materials were as described previously [10]. β ARK-PH [the GST (glutathione S-transferase) fusion protein which contains the pleckstrin homology (PH) domain of β -adrenergic-receptor kinase (β ARK) plus a C-

Abbreviations used: ADP-R, ADP-ribosylation; β ARK-PH, β -adrenergic-receptor kinase–pleckstrin homology domain (no kinase activity); DMEM, Dulbecco’s modified Eagle medium; EDIN, epidermal-cell differentiation inhibitor; FITC, fluorescein isothiocyanate; $G\beta\gamma$, $\beta\gamma$ subunit of heterotrimeric G-proteins; GTP[S], guanosine 5'-[γ -thio]triphosphate; GDP[S], guanosine 5'-[β -thio]diphosphate; HRP, horseradish peroxidase; PI 3-K, phosphoinositide 3-kinase; p85, 85 kDa subunit of PI 3-K; PI 3-K(γ), an isoform of PI 3-K that does not bind p85; PLC, phosphatidylinositol-specific phospholipase C; 3-PPI, 3-phosphorylated phosphoinositides; PtdIns, phosphatidylinositol (locants of phosphorylated forms indicated in parentheses); PtdOH, phosphatidic acid; PtdSer, phosphatidylserine; SFLLRN, Ser-Phe-Leu-Leu-Arg-Asn peptide; LPA, lysophosphatidic acid.

* To whom correspondence should be addressed.

terminal region, lacking kinase activity], purified $G\beta\gamma$ and EDIN (epidermal-cell differentiation factor) were generously provided by Dr. J. Benovic (Thomas Jefferson University), Dr. P. Casey (Duke University, Durham, NC, U.S.A.) and Dr. M. Sugai (University School of Dentistry, Hiroshima, Japan), respectively. Antibodies against $p85\alpha$, $p85\beta$ or $p85\gamma$ (monoclonal), p110 γ (polyclonal) and Rho A (polyclonal) were gifts from Dr. I. Gout (Ludwig Institute for Cancer Research, London, U.K.), Dr. R. Wetzker (Max Planck Research Group, Jena, Germany) and Dr. A. Hall (University College, London, U.K.) respectively. Guanosine 5'-[γ -thio]triphosphate (GTP[S]) and guanosine 5'-[β -thio]diphosphate (GDP[S]) were purchased from Boehringer Mannheim, and β -NAD, phosphatidylserine (PtdSer), PtdIns(4,5) P_2 , wortmannin and fluorescein isothiocyanate (FITC)-conjugated phalloidin were from Sigma Chemical Co. LY294002 was purchased from BioMol (Plymouth Meeting, PA, U.S.A.). Enhanced chemiluminescence reagents and anti-mouse or -rabbit IgG-HRP (horseradish peroxidase) antibodies were from Amersham.

PI 3-K activity in CHRF-288 cells labelled with [32 P] P_i

CHRF-288 cells obtained from Dr. Michael A. Lieberman (University of Cincinnati) were grown in suspension and prepared as described previously [5]. Briefly, cells (10^6 /ml) were washed twice with phosphate-free DMEM containing 10 mM Hepes, pH 7.6 (medium B). Serum-free cells were resuspended in medium B [(1–1.5) $\times 10^7$ cells/ml] and incubated with 0.75–1 mCi of [32 P] P_i for 1.5 h at 37 °C to achieve steady-state labelling. The labelled cells were brought to room temperature, centrifuged (900 rev./min for 5 min), resuspended in medium B [(1–1.5) $\times 10^7$ /ml] and incubated at 37 °C for 15 min. In some studies DMSO ($\leq 0.02\%$) or wortmannin (1–200 nM) was present for 15 min. Cells (500 μ l) were then incubated with 0–1 unit/ml thrombin or 0–10 μ M SFLLRN (thrombin-receptor ligand). The reactions were quenched at various times and the inositol lipids were resolved and quantified as described previously [10]. [32 P]PtdIns(3,4,5) P_3 and [32 P]PtdIns(3,4) P_2 were also deacylated and deglycerated, and the products were identified, respectively, as [32 P]Ins(1,3,4,5) P_4 and [32 P]Ins(1,3,4) P_3 as described previously [11].

PI 3-K activity in CHRF-288 cells labelled with *myo*-[2- 3 H(n)]inositol

CHRF-288 cells (15 ml) were added to 35 ml of *myo*-inositol-free DMEM (Medium C) containing 10% dialysed horse serum and grown for 3 days. These cells were split again as described above, and 0.5 mCi of *myo*-[3 H]inositol was added to each of two 50 ml flasks and the mixtures were incubated for 3 days. Simultaneously, cells were grown as for inositol-labelling studies and labelled with [32 P] P_i as described above. 3 H-labelled cells (2×10^7 /ml) were incubated with DMSO ($\leq 0.2\%$) or wortmannin (100 nM) for 15 min, and further incubated with thrombin for 0, 30 or 300 s. 32 P-labelled cells (2×10^7 /ml) were incubated for 0 or 30 s with thrombin. PtdIns(3) P , PtdIns(4) P , PtdIns(3,4) P_2 , PtdIns(4,5) P_2 and PtdIns(3,4,5) P_3 were quantified as above.

PI 3-K activity in permeabilized CHRF-288 cells

CHRF-288 cells were washed as described above and resuspended in medium B (1×10^8 cells/ml). The cells (100 μ l) were permeabilized by addition to 400 μ l of buffer containing saponin (20 μ g/ml), ATP (500 μ M) and [γ - 32 P]ATP (20 μ Ci) as described previously [2]. After 5 min incubation, buffer, thrombin (5 units/

ml), GTP[S] (2 or 20 μ M) or thrombin + GTP[S] (2 μ M) were added and further incubated for 5 min. The reactions were terminated, and phosphoinositides were extracted and quantified as described above.

PI 3-K activity in CHRF-288-cell cytosol

CHRF-288 cells were grown and washed as described above. The cells (1×10^6 /ml) were washed twice again in buffer A [2], and harvested by centrifuging at room temperature, at 900 rev./min for 5 min. The cells were resuspended (1×10^8 /ml) in buffer containing 10 mM Hepes, 1 mM EGTA, 1 mM $Na_4P_2O_7$, 2 mM Na_3VO_4 , 50 μ M dithiothreitol, 100 μ M PMSF, 200 μ M leupeptin and 5 μ g/ml of antipain, pH 7.0. CHRF-288 cells were sonicated with a micro-probe for 4×10 s, intensity setting 3 (model W-375 sonicator). The CHRF cytosol was obtained by centrifugation at 100 000 g for 90 min at 4 °C. Protein content of the cytosol was determined by the Pierce protein assay. The cytosol prepared was divided into batches and stored at -80 °C for further use. CHRF cytosol was incubated with or without EDIN (the Rho ADP-ribosylating enzyme) in the ADP-ribosylation buffer (pH 8.0) for 5 min at 37 °C, as described previously [4]. The cytosol was then assayed for PI 3-K activity with or without GTP[S] (5 μ M), with or without GDP[S] (10 μ M) with or without $G\beta\gamma$ (1 μ M) or with or without β ARK-PH (5 μ M) with PtdIns(4,5) P_2 /PtdSer as the substrate, for 5 min at 37 °C. The incubations were terminated and phosphoinositides extracted and resolved as described elsewhere [4].

Western-blot analysis of PI 3-K isoforms in CHRF-288 cells compared with platelets

Equal amounts of cytosolic proteins from CHRF-288 cells or platelets (platelet cytosol prepared as described previously [4]) were resolved by SDS/PAGE (7.5% gels) for $p85$ and $p110$, and SDS/PAGE (12.5% gels) for $p21Rho$, and transferred on to a nitrocellulose membrane. Membranes were blocked with 5% milk, and probed with monoclonal anti- $p85$ antibodies α , β and γ (each 1:2000) or with polyclonal anti-p110 γ antibody (1:200) or polyclonal anti-Rho antibody (1:3000), followed by incubation with anti-mouse IgG-HRP antibody (1:10000) for the monoclonals and with anti-rabbit IgG-HRP antibody (1:20000) for polyclonals. The blots were developed by enhanced chemiluminescence and quantified by Bio-Image scanning of the bands of interest.

Microscopic and immunofluorescence studies of CHRF-288 cells

CHRF-288 cells (2×10^5 /ml), washed free of serum, were plated on 16-well culture slides and serum-starved for 3 h before incubation with DMSO, wortmannin (100 nM) or LY294002 (50 μ M) for 15 min. The cells were then incubated with buffer, thrombin (1 unit/ml), or SFLLRN (10 μ M) for 5 min. The shape change was monitored by differential interference contrast microscopy and cells were photographed. For actin-filament staining, cells were incubated with DMSO or wortmannin for 5 min and fixed after incubation with thrombin (1 unit/ml) for 15 s or 5 min, by using 4% paraformaldehyde in sodium phosphate buffer (0.1 M), pH 7.4, for 20 min at room temperature. Cells were washed with PBS, and then the cells were permeabilized with 0.2% Triton X-100 for 5 min, and incubated with 0.1 μ g/ml FITC-labelled phalloidin for 40 min at room temperature. Cells were viewed with a Bio-Rad MRC-600 confocal microscope at a focal plane of approx. 5 μ m above the substrate and near the middle of the cell, and photographed.

Cell-proliferation studies

CHRF-288 cells ($2 \times 10^5/\text{ml}$), washed free of serum, were incubated in serum-free medium at 37°C . After 24 h, α -thrombin (1 unit/ml) or buffer was added. The cells were further incubated for 24 or 48 h. Cells were counted at each stage [5].

RESULTS

As presented in Figures 1(A) and 2(A), α -thrombin (1 unit/ml) or SFLLRN ($10 \mu\text{M}$) stimulated rapid accumulation of ^{32}P -labelled $\text{PtdIns}(3,4,5)\text{P}_3$ (up to 11- or 13-fold increase, respectively), approximately twice the maximum increases over the basal levels that we have reported for LPA or serum as agonists [5]. The levels of $\text{PtdIns}(3,4,5)\text{P}_3$ then fell to half the maximum values within 4–5 min (thrombin) or 2 min (SFLLRN). Labelled $\text{PtdIns}(3,4,5)\text{P}_3$ remained 4-fold elevated in thrombin-stimulated cells even after 10 min; however, in SFLLRN-stimulated cells, it returned to basal levels. $\text{PtdIns}(3,4)\text{P}_2$ accumulated 8.5- or 11.5-fold in 5 min, respectively, and increased with time. These two agents also caused rapid depletion of $\text{PtdIns}(4,5)\text{P}_2$ (66–72 % with thrombin/30 s; 39–44 % with SFLLRN/30 s) and increased phosphatidic acid (PtdOH) accumulation, which is used as a measure for PLC activation. By analogy with platelets and other cells, PtdOH generated in this period arises from the action of diacylglycerol kinase on diacylglycerol derived from PLC action

Figure 1 Time course and dose-response for the metabolism of ^{32}P -labelled phosphoinositides in thrombin-stimulated CHRF-288 cells

CHRF-288 cells labelled with $^{32}\text{P}\text{P}_i$ were incubated with α -thrombin (1 unit/ml) for various times (A) or (for 30 s) with varied concentrations (B). The $^{32}\text{P}\text{GroPns}$ polyphosphates of deacylated phosphoinositides were separated by HPLC, and PtdOH was separated by TLC as described in the Materials and methods section and counted for radioactivity. The results are averages $\pm$ range of a representative experiment done in duplicate. ○, $\text{PtdIns}(3,4,5)\text{P}_3$; ●, $\text{PtdIns}(3,4)\text{P}_2$; △, $\text{PtdIns}(4,5)\text{P}_2$; ▲, PtdOH. Basal values (d.p.m.) for various phosphoinositides in the time-course/dose-response experiments were: $\text{PtdIns}(3,4,5)\text{P}_3$, $2992 \pm 633/2696 \pm 119$; $\text{PtdIns}(3,4)\text{P}_2$, $6665 \pm 399/2021 \pm 93$; $\text{PtdIns}(4,5)\text{P}_2$, $(2.9 \pm 0.4) \times 10^6/(1.65 \pm 0.05) \times 10^5$; PtdOH, $(2.3 \pm 0.25) \times 10^5/(2.25 \pm 0.2) \times 10^5$.

Figure 2 Time course and dose-response for the metabolism of ^{32}P -labelled phosphoinositides in SFLLRN-stimulated CHRF-288 cells

CHRF-288 cells labelled with $^{32}\text{P}\text{P}_i$ were incubated with SFLLRN ($10 \mu\text{M}$) for various times (A) or (for 30 s) with varied concentrations (B), and lipids were quantified as in Figure 1. ○, $\text{PtdIns}(3,4,5)\text{P}_3$; ●, $\text{PtdIns}(3,4)\text{P}_2$; △, $\text{PtdIns}(4,5)\text{P}_2$; ▲, PtdOH. Basal values (d.p.m.) for various phosphoinositides in the time-course/dose-response experiments were: $\text{PtdIns}(3,4,5)\text{P}_3$, $3243 \pm 260/6563 \pm 1104$; $\text{PtdIns}(3,4)\text{P}_2$, $2542 \pm 356/9591 \pm 413$; $\text{PtdIns}(4,5)\text{P}_2$, $(1.05 \pm 0.05) \times 10^6/(5.8 \pm 0.04) \times 10^6$; PtdOH, $(2.6 \pm 0.2) \times 10^5/(4.9 \pm 0.7) \times 10^5$.

Figure 3 Inhibition by wortmannin of thrombin-stimulated 3-PPI accumulation

^{32}P -labelled CHRF-288 cells were incubated at 37°C for 5 min with 0–200 nM wortmannin before stimulation by thrombin (1 unit/ml) for 30 s. Lipids were resolved as in Figure 1. ○, $\text{PtdIns}(3,4,5)\text{P}_3$; ●, $\text{PtdIns}(3,4)\text{P}_2$.

on phosphoinositide. There was a rapid resynthesis of $\text{PtdIns}(4,5)\text{P}_2$, back to the basal levels in 1 min (thrombin) or 2 min (SFLLRN). Increases were dose-dependent (Figures 1B and 2B), reaching maximum levels for $\text{PtdIns}(3,4,5)\text{P}_3$ at 1 unit/ml (thrombin) or $10 \mu\text{M}$ (SFLLRN) concentrations, although $\text{PtdIns}(3,4)\text{P}_2$ and PtdOH had not yet reached a plateau even at $10 \mu\text{M}$ SFLLRN (Figure 2B). Similarly, increases in

Figure 4 Inhibition by wortmannin or LY294002 of thrombin-receptor-mediated shape changes in CHRF-288 cells

Serum-starved CHRF-288 cells were treated with DMSO/buffer (panels **A, B, C**), 100 nM wortmannin (panels **D, E**), or 50 μ M LY294002 (panels **F, G**) before stimulation with 10 μ M SFLLRN (panels **C, E, G**) or 1 unit/ml thrombin (panels **B, D, F**) for 5 min. The cells were photographed without fixation. Arrows indicate blebs. Bar represents 25 μ m.

[3 H]PtdIns(3,4,5) P_3 (5-fold), [3 H]PtdIns(3,4) P_2 (3.5-fold) and [3 H]PtdIns(3) P (1.6-fold) and, consistent with the activation of PLC, a decrease in [3 H]PtdIns(4,5) P_2 , were observed for *myo*-[3 H]inositol-labelled platelets that were incubated for 30 s with α -thrombin. These findings indicate that there is a net accumulation of 3-PPI in these cells upon stimulation with thrombin-receptor-directed agonist.

When CHRF-288 cells were preincubated with increasing concentrations of wortmannin (0–200 nM), agonist-stimulated PtdIns(3,4,5) P_3 and PtdIns(3,4) P_2 formations were progressively inhibited (Figure 3), with an $IC_{50} \sim 10$ nM. PtdOH accumulation

Figure 5 Blebbing and actin filament localization in thrombin-stimulated CHRF-288 cells in the presence or absence of wortmannin

CHRF-288 cells were incubated with 50 nM wortmannin (**D, F**) or DMSO (**A, B, C, E**) for 5 min before they were incubated with buffer (**A**) or α -thrombin (1 unit/ml) for 15 s (**B**) or 5 min (**C–F**). The cells were fixed and permeabilized, then treated with FITC-labelled phalloidin (1 μ g/ml) as described in the text. The labelled cells were photographed using the confocal microscope. Bar represents 10 μ m (panels **A, B, E, F**) or 20 μ m (panels **C, D**).

was not inhibited (results not shown), indicating that neither receptor function nor PLC was impaired by wortmannin.

CHRF-288 cells, when activated with 10 μ M SFLLRN or 1 unit/ml α -thrombin, showed a shape change, manifested as multiple blebblings of the plasma membrane. Cell surface blebbing could be observed within a 30 s exposure to the agonist. The blebbing was observed by differential interference contrast microscopy (Figure 4) or by confocal microscopy (Figure 5). Wortmannin pretreatment of these cells for 5–15 min, with concentrations at which it specifically inhibits 3-PPI accumulation, caused inhibition of the formation of blebs (Figures 4 and 5). The PI 3-K inhibitor LY294002 also inhibited such blebbing (Figure 4). Our results with FITC-phalloidin-stained cells (Figure 5) show further that actin filaments were not included in the membrane blebs, thereby distinguishing this morphology from lamellipod-like cell shape changes. As is true for LPA, but in contrast with serum, thrombin or SFLLRN did not promote proliferation of CHRF-288 cells (results not shown).

We observed that 20 μ M GTP[S] stimulated PtdIns(3,4,5) P_3 and PtdIns(3,4) P_2 accumulations by approx. 3-fold in saponin-permeabilized CHRF-288 cells labelled with [γ - 32 P]ATP (Figure 6), indicating the involvement of GTP-binding protein(s). Neither GTP[S] at 2 μ M nor thrombin at 5 units/ml was effective individually in stimulating PI 3-K when added to these cells; however, there was a synergistic potentiation of 3-PPI accumulation of 4–6-fold by these two agents, consistent with a role for

Figure 6 Synergistic activation of PI 3-K in permeabilized CHRF-288 cells

Saponin-permeabilized CHRF-288 cells were incubated as described in the Materials and methods section with buffer, GTP[S] ($\text{GTP}\gamma\text{S}$; 0–20 μM ; $\circ$), or thrombin ($\bullet$) and GTP[S] (2 μM) in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Lipids were extracted, resolved and quantified. (A) PtdIns(3,4,5) P_3 ; (B) PtdIns(3,4) P_2 . Results are averages $\pm$ range (d.p.m.) of an experiment in duplicate representative of three experiments. Similar results were obtained when SFLLRN was substituted for thrombin.

Table 1 Activity of PI 3-K in soluble fraction of CHRF cells

Data are expressed as percentages of basal activity (mean $\pm$ S.D. for two separate experiments). Cytosol was incubated as described, with PtdIns(4,5) P_2 as substrate. Cytosol was pre-treated for 5 min at 37 $^{\circ}\text{C}$ with βNAD^+ (200 μM) with or without EDIN (20 $\mu\text{g}/\text{ml}$). Concentrations used: GTP[S], 10 μM ; $\text{G}\beta\gamma$, 1 μM ; $\beta\text{ARK-PH}$, 5 μM . $\beta\text{ARK-PH}$ or EDIN was without effect on basal activity, as was true for GDP[S] (10 μM).

Inhibitor	PI 3-K activity (% of basal)		
	Agonist ... GTP[S]	$\text{G}\beta\gamma$	GTP[S] + $\text{G}\beta\gamma$
None	285 $\pm$ 11	308 $\pm$ 13	578 $\pm$ 6
EDIN	122 $\pm$ 13	309 $\pm$ 5	—
$\beta\text{ARK-PH}$	286 $\pm$ 3	105 $\pm$ 3	—

receptor occupancy in enhancing GTP binding to G-protein and thereby promoting a downstream PI 3-K effect.

That there are two forms of PI 3-Ks, regulated by the small GTP-binding protein Rho and by $\text{G}\beta\gamma$ subunits of heterotrimeric G-protein, is indicated by Tables 1 and 2. The cytosolic fraction of unactivated CHRF-288 cells contained PI 3-K activity (Table 1) that could be stimulated $\sim$ 2.9-fold by GTP[S] or 3.1-fold by $\text{G}\beta\gamma$, and was unaffected by GDP[S]. Incubation of the soluble CHRF cell fraction with EDIN, an enzyme known to ADP-ribosylate and inactivate Rho [4], caused virtually complete inhibition of GTP[S]-stimulatable, but no inhibition of $\text{G}\beta\gamma$ -stimulated, PI 3-K activity. Similarly, when $\beta\text{ARK-PH}$, which binds to $\text{G}\beta\gamma$, was added to the cytosolic fraction, it caused a complete inhibition of $\text{G}\beta\gamma$ -stimulatable PI 3-K activity, without

Table 2 Relative quantities of proteins in platelet or CHRF soluble fractions involved in PI 3-K activity

Proteins from high-speed supernatants of platelet or CHRF lysates were applied to SDS/polyacrylamide gels (equal amounts were applied per well) and resolved as described. Proteins were detected by Western blotting/enhanced chemiluminescence and quantified by densitometric scanning. Numbers in parentheses represent ratios of Rho/average total p85. 'General p85' refers to p85 detected by a mixture of monoclonal antibodies for all three forms. 'Σp85' refers to the sum of $\alpha + \beta + \gamma$ isoforms, detected separately. Results were obtained in two separate experiments (one in duplicate), in the linear range of detection.

	Platelet	CHRF	CHRF/platelet
p85 (α)	8.7	7.5	0.86
p85 (β)	3.9	12.2	3.1
p85 (γ)	0.06	0.19	3.2
Σp85	12.6	19.9	1.6
General p85	12.4	19.2	1.5
Rho	5.3 $\pm$ 0.4 (0.42)	3.5 $\pm$ 0.0 (0.18)	0.66
PI3-K (γ)	0.44 $\pm$ 0.05	0.29 $\pm$ 0.03	0.66

affecting GTP[S]-stimulated PI 3-K. The presence of both $\text{G}\beta\gamma$ and GTP[S] led to essentially additive stimulation. These results clearly demonstrate the functional presence in CHRF-288 cells of the two isoforms of PI 3-K shown to be activated in platelets [4].

As an extension of these studies, we have looked at the presence of different isoforms of p85 (a regulatory subunit of heterodimeric PI 3-K) and of PI 3-K(γ) (which does not bind p85) by quantitative Western blotting (Table 2). Our results indicate that the relative amounts of p85 β and p85 γ subunits are 3-fold higher in CHRF-288 cells compared with those in platelets, whereas amounts of p85 α are approximately equal in the cell types. In contrast, platelets are richer in Rho and PI 3-K(γ) than are CHRF-288 cells, and the ratio of Rho to total p85 for CHRF-288 cells is 43 % of that for platelets.

DISCUSSION

In the present studies we have examined the regulation of 3-PPI accumulation and morphological changes induced by thrombin-receptor-directed agonists in CHRF-288, a cell line derived from platelet progenitor cells. Our kinetic results for 3-PPI accumulation using SFLLRN or thrombin indicate that PtdIns(3,4,5) P_3 accumulates rapidly and transiently, most likely as a result of phosphorylation of PtdIns(4,5) P_2 by PI 3-K, as reported for platelets [11]. PtdIns(3,4) P_2 , which appears after a slight delay, may arise from hydrolysis of PtdIns(3,4,5) P_3 or by PtdIns(4) P 3-kinase activation in these cells. These results are similar to those observed in thrombin-or SFLLRN-stimulated platelets [2,10,11]. Activation of PI 3-K by thrombin-receptor-directed ligand is greater than that induced by serum or LPA. That wortmannin inhibits PI 3-K, without affecting receptor function or other lipid-metabolizing enzymes, is clear from our results in which thrombin-stimulated formation of PtdOH is not inhibited and levels of PtdIns(4,5) P_2 are not affected (results not shown) by exposure of CHRF-288 cells to wortmannin.

α -Thrombin or SFLLRN also causes rapid shape changes, manifested as blebbing of the CHRF-288 cell membrane. Neither LPA nor serum causes such dramatic changes, as monitored by differential interference contrast microscopy. The blebs do not contain any phalloidin-detectable actin filaments, thereby distinguishing them from lamellipod-like structures. Exposure to thrombin seems to create a destabilization or disconnection of the plasma membrane with respect to the cytoskeletal matrix,

rather than inducing an active pseudopodal outgrowth as a function of actin polymerization in CHRF-288 cells. Formation of blebs can be inhibited by wortmannin at concentrations where wortmannin specifically inhibits PI 3-K or by LY294002 [9] under similar conditions. These results are consistent with the involvement of PI 3-K in this cell response. Blebbing may be dependent on PtdIns(3,4,5) P_3 [or PtdIns(3,4) P_2]-activated protein kinase, rather than on direct interaction of such phosphoinositides with cytoskeletal proteins. In terms of mass (for stoichiometric interactions), the amount of 3-PPI produced in platelets and CHRF-288 cells is relatively minor in comparison, e.g., with the levels of PtdIns(4,5) P_2 . We have observed, however, that PtdIns(3,4,5) P_3 can stimulate protein phosphorylation in platelets [12]. Although CHRF-288 cells do not form active $\alpha_{11b}\beta_3$ and aggregate in response to activation by thrombin [13], there may be some fundamental parallels, like blebbing/microvesicle formation, relevant to formation of pro-coagulant activity in platelets. It is possible that one of the pathways for microvesicle formation in activated platelets [14,15] may involve two stages, i.e. PI 3-K-dependent, wortmannin-inhibitable, membrane blebbing, followed by shedding of blebs.

Our studies using saponin-permeabilized CHRF-288 cells have shown that GTP-binding protein(s) plays a role in regulating PI 3-K activity, as in the case of platelets [2]. We also observed a synergistic potentiation of PI 3-K activity when thrombin and GTP[S] were used, indicating a role for receptor occupancy in GTP binding to G-protein, an effect which typifies receptor-activated heterotrimeric G-proteins. Consistent with our results in platelets [4], we have observed the presence of two different forms of PI 3-K in CHRF-288 cells, respectively dependent on functional Rho and $G\beta\gamma$. These results are based on the inhibitory effects of EDIN (which ADP-ribosylates Rho) and β ARK-PH (which binds to $G\beta\gamma$) on PI 3-K activity. Both the $G\beta\gamma$ and Rho routes of activation are dependent on GTP. The former is via GTP-dependent dissociation of $G\alpha$ from $G\beta\gamma$, and the latter is via the binding of GTP to the small G-protein Rho.

That all the PI 3-K isoforms described for platelets are found in CHRF-288 cells is also demonstrated from our Western-blotting data. We observed that these cells contain α , β and γ isoforms of p85, as seen in platelets, but in somewhat different proportions. Also, platelets contain higher amounts of Rho and PI 3-K(γ) and have a higher ratio of Rho to total p85. Since conventional catalytic p110 (α or β) is bound to p85 α , β (and possibly γ), such subunits play an important role in localizing this well-characterized PI 3-K in the cells. Binding preferences of p85 α versus p85 β and p85 γ are as yet unknown, although selectivity in phosphorylation of p85 β versus p85 α as a function of T-cell activation by TCR/CD3 complex or protein kinase C has been reported [16]. Thus localization and/or activation of p85/PI 3-K in platelets compared with CHRF-288 cells may be due to the types of p85 present. Localization of PI 3-K by p85 to the occupied receptor has already been shown to be regulated by exposure of certain cells to platelet-derived growth factor [17], since p85 binds via its two SH2 domains to the tyrosine-phosphorylated receptor. Other possible targets may involve exposure of proline-rich domains of p85 to the SH3 domain of src-family proteins [18]. Neither the thrombin receptor nor the LPA receptor is a tyrosine-autophosphorylating kinase, although tyrosine kinases are activated in response to occupancy of these receptors. In platelets, p85/PI 3-K is activated in a Rho-dependent (and most likely tyrosine phosphorylation-dependent) manner when platelets are exposed to thrombin. It remains to be

determined whether the activation is limited to one species of p85-containing PI 3-K.

Since both thrombin receptors and LPA receptors are known to activate heterotrimeric G-proteins [19,20], it is probable that $G\beta\gamma$ is made available in CHRF-288 cells exposed to these agonists, enabling activation of PI 3-K(γ). Serum growth factors such as platelet-derived growth factor, which activates p85/PI 3-K, are not known to activate heterotrimeric G-proteins. LPA [5] and thrombin, in contrast with serum, potentially stimulate PI 3-K without promoting a proliferative response from CHRF-288 cells, consistent with our conclusion that accumulation of 3-PPI is not itself sufficient for growth promotion. It is not known whether in CHRF-288 cells LPA and thrombin stimulate p85/PI 3-K, which serum peptide growth factors would be expected to do. It is possible that localization of activated PI 3-K and/or the types of PI 3-K activated play a role in proliferation. This would imply that the site of generation of membrane-anchored second messenger [most likely PtdIns(3,4,5) P_3 , but also potentially PtdIns(3,4) P_2], as well as the amount, could determine the biological response. Conceivably, factors stimulated by serum, not monitored in our studies and not stimulated by LPA and thrombin, in conjunction with 3-PPI, promote proliferation. These are topics of current interest in our laboratory.

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