0026-895X/95/030532-08$3.00/0

MOLECULAR PHARMACOLOGY, 48:532–539 (1995).

Regulation of Phosphoinositide-3-kinase by G Protein βγ Subunits in a Rat Osteosarcoma Cell Line

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Received April 26, 1995; Accepted June 5, 1995

SUMMARY

Rat osteosarcoma 17/2.8 cells (Ros 17/2.8 cells) were labeled with $[^{32}P]PO_4^{2-}$, and their levels of inositol lipids were determined after stimulation with thrombin. Thrombin stimulated a pertussis toxin-sensitive rapid accumulation of phosphatidylinositol-3,4,5-trisphosphate [PtdIns(3,4,5)P_3] with lesser increases in levels of phosphatidylinositol-3,4-bisphosphate [PtdIns(3,4)P_2] and phosphatidylinositol-3-phosphate [PtdIns3P] that were slower in onset. Ros 17/2.8 cell homogenates contained phosphatase activities that hydrolyzed PtdIns(3,4,5)P_3 to PtdIns(3,4)P_2 and PtdIns3P. Phosphoinositide-3-kinase activity was determined in Ros 17/2.8 cell homogenates using exogenously provided PtdIns(4,5)P_2. Guanosine-5′-3-*O*-(thio)triphosphate caused an approximately 3-fold increase in phosphoinositide-3-kinase activity in a manner that was blocked by high concentrations of guanosine-5′-2-*O*-(thio)diphosphate. Purified bovine brain G protein βγ subunits also increased phosphoinositide-3-kinase activity modestly in Ros 17/2.8 cell homogenates. Ros 17/2.8 cell homogenates contained phosphatase activities that sequentially dephosphorylated PtdIns(3,4,5)P_3 to PtdIns(3,4)P_2 and PtdIns3P. Two peaks of phosphoinositide-3-kinase activity were resolved by anion exchange chromatography of a Ros 17/2.8 cell cytosolic extract. The later elution of these was selectively activated by βγ subunits (16-fold activation with 16 μM βγ subunits). Half-maximal effects of the βγ subunits were observed at a concentration of 0.6 μM, and activation was blocked by preincubation of the βγ subunits with an excess of recombinant $G_{i\alpha 2}$. βγ Subunits did not activate the p85α/p110β form of phosphoinositide-3-kinase purified from sf9 cells after expression with the use of baculovirus vectors.

Inositol lipid kinases are a family of enzymes that catalyze the ATP-dependent phosphorylation of the three *myo*-inositol-containing phospholipids: PtdIns,[1] PtdIns4P_2, and PtdIns(4,5)P_2 (1). The primary substrate of the PLC-driven signal-generating system, PtdIns(4,5)P_2, is synthesized by sequential phosphorylation of PtdIns via the intermediate PtdIns4P. These reactions are catalyzed by two distinct ATP-dependent enzymes, PtdIns 4-kinase and PtdIns4P 5-kinase (2). Cells also contain polyphosphoinositide derivatives of PtdIns, PtdIns4P, and PtdIns(4,5)P_2 in which the D-3 OH group is phosphorylated (3–7). These "3-phosphorylated" inositol lipids, PtdIns3P, PtdIns(3,4)P_2, and PtdIns(3,4,5)P_3, can be synthesized *in vitro* by phosphorylation of PtdIns, PtdIns4P, and PtdIns(4,5)P_2, and these reactions are catalyzed by members of a growing family of phosphoinositide-3-kinases, some of which are receptor-regulated enzymes (8, 9). Many cells challenged with a variety of different agonists exhibit rapid increases in levels of the 3-phosphorylated inositol lipids, most notably PtdIns(3,4,5)P_3 (4–7; see Refs. 8 and 9 for reviews). Several lines of molecular genetic and biochemical evidence supports the idea that the 3-phosphorylated inositol lipids serve as membrane-associated second messengers (9). For example, mutations of receptors that block phosphoinositide-3-kinase activation, experiments with the potent phosphoinositide-3-kinase inhibitor wortmannin, and microinjection studies with neutralizing phosphoinositide-3-kinase antibodies implicate phosphoinositide-3-kinase and its products in regulation of cell growth, chemotaxis, intracellular trafficking of the platelet-derived growth factor receptor, respiratory burst generation in neutrophils, insulin-stimulated glucose transport in adipocytes, and the membrane ruffling response of fibroblasts to mitogens (10–14). The biochemical mechanisms by which phosphoinositide-3-kinase and its products mediate these processes are unclear. Two recent studies indicate that *in vitro*, PtdIns(3,4,5)P_3 is a potent direct activator of the δ, ε, η, and possibly ζ members of the Ca^{2+}-insensitive phospholipid-dependent protein kinase C family (15,16).

At our current level of understanding, the mechanisms used by cell surface receptors to regulate phosphoinositide-

ABBREVIATIONS: PtdIns, phosphatidylinositol (positions of phosphate group substituents indicated in parentheses); Ros 17/2.8 cells, rat osteosarcoma 17/2.8 cells; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; PLC, inositol lipid-specific phospholipase C; HPLC, high performance liquid chromatography; DMEM, Dulbecco's modified Eagle's medium; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

3-kinase activity display an obvious parallel to those recently deduced for receptor regulation of PLC (17). Ligands acting on representatives of two broad classes of cell surface receptors, receptor tyrosine kinases and G protein-coupled receptors, stimulate phosphoinositide-3-kinase in intact cells. The phosphoinositide-3-kinase target of receptor tyrosine kinases is a heterodimer composed of an 85-kDa regulatory subunit [p85] and a 110-kDa catalytic subunit [p110] (18). Three isoforms of p85 and two isoforms of p110 have been described (19–23). p85 is an SH2 domain-containing adapter protein that binds the phosphoinositide-3-kinase heterodimer to tyrosine phosphorylated receptors (or, in the case of the insulin receptor, to the receptor substrate IRS-1). Activation of p85/p110 phosphoinositide-3-kinase by receptor tyrosine kinases may result from an allosteric effect of phosphotyrosine binding to the SH2 domains of p85, from a translocation of the enzyme from the cytosol to the plasma membrane site of its lipid substrates, or from alterations in the phosphotyrosine or phosphoserine content of the p110 subunit (8, 9). The low-molecular-weight G proteins Rho and Ras are also implicated as regulators of phosphoinositide-3-kinase (24, 25).

G proteins are heterotrimeric proteins with an $\alpha\beta\gamma$ subunit structure that function as GTP-regulated intermediaries in the coupling of cell surface receptors to intracellular effectors. Functional classification of G protein oligomers has been defined by their α subunits, but it is clear that both the α and the tightly associated $\beta\gamma$ subunit dimers play direct regulatory roles in receptor-effector coupling (26). Stimulation of five different G protein-coupled receptors (the platelet thrombin and thromboxane A_2 receptors and neutrophil receptors for chemotactic peptides, platelet-activating factor, and purinergic agonists) increases phosphoinositide-3-kinase activity (4, 6, 7, 9). An extensive series of studies has established that in a variety of myeloid-derived cells, rapid activation of phosphoinositide-3-kinase by G protein-coupled receptors is a second messenger- and tyrosine kinase-independent process (7, 27, 28). In one of the cell lines studied, U937 cells, this process apparently involves direct activation of an unidentified form of phosphoinositide-3-kinase by G protein $\beta\gamma$ subunits (29). Immunological and chromatographic evidence suggest that this is distinct from the known p85/p110 heterodimers. Human platelet cytosol also contains a $\beta\gamma$ subunit-stimulated phosphoinositide-3-kinase activity that may be closely related to the heterodimeric phosphoinositide-3-kinase enzyme targets of receptor tyrosine kinases (30).

We describe the kinetics of rapid changes in inositol lipid levels in a rat osteosarcoma cell line (Ros 17/2.8 cells) during stimulation with thrombin. Our results indicate that thrombin stimulates a rapid accumulation of PtdIns(3,4,5)P_3 in these cells and that receptor-mediated accumulation of PtdIns(3,4)P_2 and PtdIns3P is most likely a consequence of phosphatase-catalyzed hydrolysis of PtdIns(3,4,5)P_3. Assays with broken cell systems and partially purified proteins implicate a G protein in thrombin receptor regulation of Ros 17/2.8 cell phosphoinositide-3-kinase activity, and we report that Ros 17/2.8 cells contain a phosphoinositide-3-kinase activity that is activated by purified G protein $\beta\gamma$ subunits *in vitro*.

Experimental Procedures

Materials. Unless otherwise noted, reagents were obtained from conventional sources. Adenine, guanine nucleotides, and their synthetic analogues were obtained from Boeringer Mannheim. Radioisotopes were obtained from ICN and American Radiolabeled Chemicals. Reagents for SDS-PAGE and immunoblotting were obtained from GIBCO-BRL and Bio-Rad. PtdIns(4,5)P_2 and PtdIns4P were purified from a Folch fraction of bovine brain lipids (Sigma Chemical Co.) by anion exchange HPLC on an Econosphere-NH_2 column (10 × 250 mm, Alltech Associates) eluted with a linear ammonium acetate gradient (0–1 M in $CHCl_3$/MeOH/H_2O 20:9:1. All other phospholipids were obtained from Avanti Polar Lipids. [3-^{32}P]PtdIns(3,4,5)P_3 was prepared by phosphorylation of PtdIns(4,5)P_2 with a preparation of the recombinantly expressed p110β isoform of the phosphoinositide-3-kinase catalytic subunit. The lipid was isolated by anion exchange HPLC as described for the unlabeled phosphoinositides except that an analytical (5 × 250 mm) column was used with a gradient of 0–1.2 M ammonium acetate, fractions were collected in polypropylene tubes, and phosphatidylserine (10 μg) was added to the pooled fractions before extraction by the addition of 3 M HCl and MeOH to give a final ratio of $CHCl_3$/MeOH/H_2O of 1:1:0.9. After mixing and centrifugation, the lower phase was removed, and the upper phase was extracted once with a synthetic lower phase, which was combined with the lower phase from the first extraction. The combined lower phases were neutralized with 20% NH_4OH in MeOH, dried under nitrogen, and used in assays as described later. [^{3}H]PtdIns, [^{3}H]PtdIns4P, and [^{3}H]PtdIns(4,5)P_2 were purified from extracts of turkey erythrocytes that had been labeled with [^{3}H]inositol by anion exchange HPLC as described for their unlabeled counterparts. Purified human α-thrombin (approximately 3500 units/mg, 1 nmol = 0.1 unit/ml) was generously provided by Dr. J. Jesty, Stony Brook Health Sciences Center, Stony Brook, NY.

Expression and purification of the p85α/p110β phosphoinositide-3-kinase heterodimer. cDNAs encoding p85α and p110β were obtained from Drs. P. Hu and J. Schlessinger, Department of Pharmacology, New York University Medical Center. Recombinant baculoviruses harboring these cDNAs were generated by standard methods. A 500-ml suspension culture of sf9 cells (10^6 cells/ml) was doubly infected at a multiplicity of infection of 5 for each virus. The cells were harvested and washed once with and resuspended in 10 ml of phosphate-buffered saline before nitrogen cavitation in a 40-ml Parr cell disruption bomb. After a 30-min equilibration at 1100 psi, the cell suspension was discharged into buffer 1 (40 ml of 25 mM Tris, pH 7.5, 2 mM EDTA, 2 mM EGTA, and 0.1 mM dithiothreitol containing 0.1 mM benzamidine and 0.1 mM phenylmethylsulfonyl fluoride). The homogenate was centrifuged at 50 × 10^3 × g_{av} for 1 hr, and the supernatant was loaded onto an 8.6-ml Pharmacia Source 15Q anion exchange column (1 × 11 cm) at a flow rate of 1 ml/min. Bound proteins were eluted with a 160-ml linear gradient of 0–60% buffer 2 (buffer 1 containing 1 M NaCl). Fractions containing phosphoinositide-3-kinase activity were pooled together (12 ml), and diluted 10-fold with buffer 3 (25 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 6.5, 2 mM EDTA, 2 mM EGTA, 0.1 mM dithiothreitol containing 0.1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride), and applied to a 1-ml Pharmacia Source 15S cation exchange column (0.5 × 1.3 cm) at a flow rate of 0.5 ml/min. Bound protein was eluted with a 15-ml linear gradient of 0–30% buffer 4 (buffer 3 containing 1 M NaCl).

Purification of G proteins. G protein $\beta\gamma$ subunits were isolated from cholate extracts of bovine brain membranes by sequential anion exchange, gel filtration, and hydrophobic interaction chromatography according to the methods of Sternweis and Robishaw (31), substituting octyl sepharose (Pharmacia) for the latter step. After activation with aluminum fluoride, $\beta\gamma$ subunits were further resolved from α subunits, concentrated, and exchanged into various detergents as necessary by repeat anion exchange chromatography on a 1-ml Pharmacia Source 15Q column eluted with a gradient of NaCl.

The βγ subunit preparations were pure as assessed by SDS-PAGE with silver staining and by Western blotting with antisera against a variety of G protein α subunits and did not contain measurable [^{35}S]GTPγS binding activity when subjected to a standard assay procedure.[1] $G_{i\alpha 2}$ was expressed in sf9 cells using a baculovirus vector generated from a cDNA provided by Dr. Gary L. Johnson, National Jewish Hospital, Denver, CO. The recombinant protein was purified from lubrol extracts of sf9 cell membranes.[2]

Cell culture. Ros 17/2.8 cells were obtained from Dr. C. C. Malbon, Department of Pharmacological Sciences, Stony Brook Health Sciences Center. The cells were cultured in DMEM supplemented with 10% fetal calf serum containing antibiotic and antifungal agents. Confluent monolayers of cells were used for all experiments. For some experiments, the cells were cultured overnight in the presence of 600 ng/ml pertussis toxin.

Measurement of inositol lipid levels in intact Ros 17/2.8 cells. Dishes (100 mm diameter) of confluent Ros 17/2.8 cells were washed once in serum-free DMEM and then incubated in serum-free DMEM for 16 hr. The cells were washed once in phosphate-free DMEM and then labeled with [^{32}P]$P0_4^{2-}$ (0.1 mCi/ml, 3 ml/dish) for 90 min. The cells were then washed once in phosphate-free DMEM, and the dishes were transferred to a 37° water bath. The cells were stimulated by the addition of thrombin (10 nM), and assays were terminated by the rapid aspiration of the medium and the addition of 2 ml of ice-cold 3 M HCl. The dishes were placed on ice, the cells were scraped up, and the lysates obtained transferred to a 50-ml polypropylene centrifuge tube. The dishes were washed with an additional 2 ml of ice-cold 3 M HCl, and this washing was combined with the lysate. Then, 30,000 dpm of [^{3}H]PtdIns(4,5)P_2 in 15 μl of $CHCl_3$/MeOH/H_2O (20:9:1) was added to the extract to act as a recovery standard and internal marker for the subsequent analyses. Lipids were extracted by the addition of 15 ml of $CHCl_3$/MeOH/concHCl (40:80:1). The tubes were mixed thoroughly by vortexing and allowed to stand at room temperature for 10 min. After the addition of another 5 ml of $CHCl_3$ and 5 ml of 0.1 M HCl, the tubes were vortexed and centrifuged at 1000 × g_{av} to separate the aqueous and organic phases. The lower organic phase was removed to a 50-ml polypropylene tube, and the upper phase was extracted once with 5 ml of a synthetic lower phase. This lower-phase wash was combined with the lower phase from the initial extraction. These pooled lower phases were washed with 20 ml of a synthetic upper phase, and after vortexing and centrifugation, the lower phase was carefully removed to a 15-ml polypropylene tube. This lipid extract was dried under vacuum, and the lipids were deacylated by the addition of 5 ml of methylamine reagent according to the procedure described. The water-soluble lipid headgroups released were extracted, dried, and resuspended in 2 ml of water for chromatographic analysis by anion exchange HPLC. Our separations used a Whatman Partisil 5 SAX column (4.6 × 110 mm) fitted with a Whatman pellicular anion exchange guard cartridge. The column was eluted at 1 ml/min with a complex gradient generated by mixing solvent A/Milli-Q water and solvent B/2 M $(NH_4)_2PO_4$, pH 3.8, in Milli-Q water. The gradient consisted of 10 min at 0% B and then a linear 30-min increase to 13% B, followed by a linear 30-min increase to 50% B. The column was then washed with 100% B for 10 min and equilibrated with solvent A for 30 min before injection of a sample. We collected fractions of eluant every 0.5 min. Radioactivity in these fractions was determined by liquid scintillation counting after the addition of 1 ml of MeOH/H_2O (1:1) and 4 ml of Packard Insta-Gel scintillant. Counting was performed with a Wallac model 1409 liquid scintillation counter set up for dual-label ^{3}H/^{32}P detection using the spectral analysis method for efficiency correction and the library of spectra provided by the manufacturer. Compounds were identified by reference to authentic chromatographic standards prepared enzymatically (in the case of PtdIns3P, PtdIns(3,4)P_2, and PtdIns(3,4,5)P_3) or obtained as described in Materials. The data shown were corrected for recovery of the internal [^{3}H]PtdIns(4,5)P_2 standard.

Exogenous substrate phosphoinositide-3-kinase and phosphatase assays. Unilamellar phospholipid vesicles were prepared by probe sonication of dried films of phosphoinositide/ phosphatidylserine (molar ratio, 1:10) in 10 mM HEPES, pH 7.5. Phosphoinositide-3-kinase assays contained 100 μM total phospholipid and 10–100 μM ATP with 10–100 μCi of [γ-^{32}P]ATP. The assay buffer contained final concentrations of 20 mM HEPES, pH 7.4, 1 mg/ml bovine serum albumin, 1 mM dithiothreitol, 115 mM KCl, 4.2 mM $MgCl_2$, 2 mM EGTA, and $CaCl_2$ to give varying free Ca^{2+} concentrations. Standard assay conditions contained a calculated Ca^{2+} concentration of 1 μM. For investigations of G protein effects on phosphoinositide-3-kinase activity, the incubations contained 0.1% cholate. G proteins were added to the incubations from concentrated solutions (usually 2–5 mg/ml) in buffer containing 1% cholate. Assay components were assembled on ice in 1.5-ml microfuge tubes and then transferred to a 37° water bath for 5 min before the addition of the source of enzyme activity. Reactions were terminated by the addition of 375 μl of $CHCl_3$/MeOH/HCl followed by 125 μl of $CHCl_3$ and 125 μl of 0.1 M HCl. The tubes were capped, vortexed vigorously, and centrifuged to separate the phases. The lower phase was carefully removed, and the upper phase was re-extracted with 200 μl of synthetic lower phase. The lower phases were combined, washed with 400 μl of synthetic upper phase, and dried under vacuum. Reaction products were analyzed by either thin layer chromatography on oxalate/EDTA-impregnated silica plates (24) or by deacylation and anion exchange HPLC as described. Thin layer chromatography analyses were performed with plastic-backed plates, and the products were identified by autoradiography and quantified by liquid scintillation counting after excision from the plate or, in some cases, with a beta-imager.

For analyses of phosphoinositide-phosphatase activities, the same assay buffer was used, omitting the radioactive ATP. Reactions were terminated by the addition of 1 ml of the methylamine deacylation reagent. Deacylation and extraction of the glycerophosphoinositols were performed as described. The products were analyzed by anion exchange HPLC.

Protein separations. Ros 17/2.8 cells were grown in 225-cm^2 flasks. Monolayers of confluent cells were detached by washing in PBS containing 1 mM EDTA. Detached cells from five flasks were recovered by centrifugation and resuspended in 10 ml of buffer 1 before disruption by sonication. The resultant homogenate was centrifuged at 50 × 10^3 × g_{av} for 30 min. The supernatant was diluted to 40 ml with buffer 1 and applied to a 1.3-ml Pharmacia Source 15Q anion exchange column that had been equilibrated with buffer 1 at a flow rate of 0.5 ml/min. After loading was complete and the absorbance of the eluant had returned to base-line levels, the column was washed with an additional 10 ml of buffer A, and then bound protein was eluted with a 20-ml linear gradient of 0–60% buffer followed by a linear 10-ml gradient of 60–100% buffer 2, again at a flow rate of 0.5 ml/min. The eluant was collected as 0.5-ml fractions, and phosphoinositide-3-kinase activity of these fractions was determined as described.

Results

Activation of phosphoinositide-3-kinase by thrombin in intact Ros 17/2.8 cells. Thrombin stimulated a rapid and transient increase in levels of PtdIns(3,4,5)P_3 in Ros 17/2.8 cells (Fig. 1). This increase was maximal at 30 sec after administration of agonist. PtdIns(3,4,5)P_3 levels fell steadily in the continued presence of agonist. Levels of PtdIns(3,4)P_2 were also increased by thrombin but lagged behind the rise in PtdIns(3,4,5)P_3. Although increases in PtdIns(3,4,5)P_3 levels could be detected 15 sec after stimulation, at this time point PtdIns(3,4)P_2 levels were barely elevated above basal, reaching a maximum at 1 min and then falling steadily in the

[1] A. J. Morris and J. M. Jenco, unpublished observations.
[2] A. J. Morris, C. M. Moxham, and C. C. Malbon, unpublished observations.

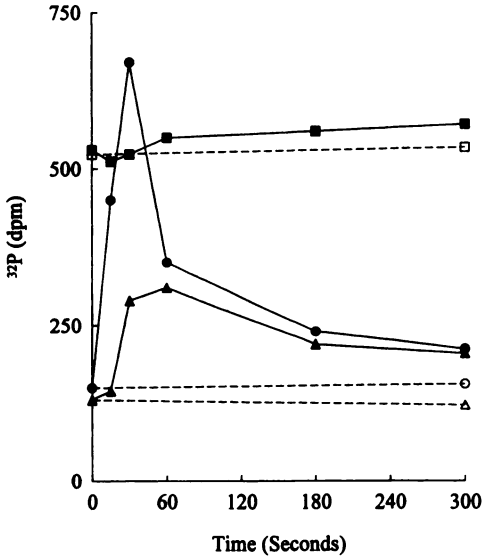

Fig. 1. Activation of phosphoinositide-3-kinase by thrombin in intact Ros 17/2.8 cells. Plates of serum-starved Ros cells were labeled with $[^{32}P]PO_4^{2-}$ as described in the text. The cells were stimulated with thrombin, and inositol lipid species were quantified after deacylation and resolution of glycerophosphoinositol headgroups by anion exchange HPLC using procedures described in the text. ○, unstimulated PtdIns(3,4,5)P_3; ●, thrombin-stimulated PtdIns(3,4,5)P_3; △, unstimulated PtdIns(3,4)P_2; ▲, thrombin stimulated PtdIns(3,4)P_2; □, unstimulated PtdIns3P; ■, thrombin stimulated PtdIns3P.

continued presence of agonist. By contrast, thrombin did not stimulate a rapid accumulation of PtdIns3P, and accumulation of this lipid was extremely modest over the time course studied.

Overnight treatment of Ros 17/2.8 cells with pertussis toxin completely abolished the rapid rise in PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 levels, suggesting the involvement of a G_i protein in thrombin receptor regulation of phosphoinositide-3-kinase in these cells (Table 1).

Regulation of phosphoinositide-3-kinase in Ros 17/2.8 cell homogenates. To investigate the biochemical mechanisms underlying thrombin receptor regulation of phosphoinositide-3-kinase in Ros 17/2.8 cells, we undertook studies of the regulation of this activity in Ros 17/2.8 cell homogenates using exogenously provided substrates presented as components of mixed phospholipid vesicles. Phosphoinositide-3-kinase activity was readily detectable in these extracts with PtdIns, PtdIns4P, or PtdIns(4,5)P_2 as substrate. With PtdIns(4,5)P_2 as substrate, accumulation of PtdIns(3,4,5)P_3 was approximately linear over the first 2 min of these incubations (Fig. 2A). Inclusion of 10 μM GTPγS in these assays caused a 3-fold increase in the initial rate of phosphorylation of PtdIns(4,5)P_2 (Fig. 2A). Concurrent with these experiments, we prepared radiolabeled PtdIns(3,4,5)P_3 and examined the metabolism of this compound when added to Ros 17/2.8 cell homogenates. The results are illustrated in Fig. 2B. We observed rapid degradation of PtdIns(3,4,5)P_3 to PtdIns(3,4)P_2. This latter compound was more slowly degraded to produce PtdIns3P. The slowing of PtdIns(3,4,5)P_3 accumulation observed at later times in the assays illustrated in Fig. 2A therefore appears to be attributable to the PtdIns(3,4,5)P_3 5-phosphatase in the homogenates, and we observed generation of PtdIns(3,4)P_2 at these later times (data not shown). We next explored the specificity of this stimulatory effect of GTPγS and evaluated the role of some purified subunits of heterotrimeric G proteins in this process. These assays were identical to those illustrated in Fig. 2A except that the medium contained 0.1% cholate and the assay time was 90 sec. Under these conditions, 10 μM GTPγS elicited a 2.5-fold increase in phosphoinositide-3-kinase activity, and this effect could be blocked completely by 50 μM guanosine 5′-2-*O*-(thio)diphosphate. Inclusion of 1.6 μM purified G protein βγ subunits produced a 1.9-fold activation of phosphoinositide-3-kinase in these homogenates. Preincubation of the βγ subunits with an excess of purified recombinant α_{i2} (final concentration, 3.9 μM) in the presence of GDP and Mg^{2+} before addition to the assay essentially ablated this stimulatory effect of the βγ subunits (Fig. 3). GTPγS-loaded α_{i2} had no effect on phosphoinositide-3-kinase activity when added to the homogenates (not shown).

Taken together, these results suggest a role for G proteins in regulation of Ros 17/2.8 cell phosphoinositide-3-kinase. The effects of βγ subunits implicate heterotrimeric G proteins as a potential locus of this effect. Further experiments were undertaken to evaluate the role of G protein βγ subunits in activation of Ros 17/2.8 cell phosphoinositide-3-kinase activity.

Separation of two Ros 17/2.8 cell cytosolic phosphoinositide-3-kinase activities by anion exchange chromatography. When Ros 17/2.8 cell homogenates were fractionated by ultracentrifugation, >90% of phosphoinositide-3-kinase activity was recovered in the soluble fraction. This material was subjected to anion exchange chromatography on a Source 15Q column, and the fractions that were collected were assayed for phosphoinositide-3-kinase activity with PtdIns(4,5)P_2 as substrate. Each fraction was assayed in the presence and absence of 1 μM βγ subunits. We observed two peaks of phosphoinositide-3-kinase activity in the column eluant. The earlier elution of these was insensitive to βγ subunits, whereas the later eluting activity exhibited a marked activation by βγ subunits (Fig. 4).

Activation of this phosphoinositide-3-kinase preparation was dependent on the concentration of βγ subunits in the incubation. The effect of βγ subunits was not completely saturable over the concentration range investigated (the concentration of our βγ subunit preparations and considerations of the amount of detergent that could be tolerated in the assay system precluded investigating effects of higher βγ subunit concentrations). We observed a 16-fold increase in phosphoinositide-3-kinase activity with 16 μM βγ subunits. Assuming this to be a near-maximal effect, half-maximal activation was observed at approximately 0.6 μM βγ sub-

TABLE 1

Effect of pertussis toxin on thrombin receptor stimulation of phosphoinositide-3-kinase in ros 17/2.8 cells

Ros 17/2.8 cells were serum starved and incubated with 600 ng/ml pertussis toxin overnight. The cells were labeled with $[^{32}P]PO_4^{2-}$ as described in the text and stimulated with thrombin for 30 sec, and inositol lipid species quantified after deacylation and resolution of glycerophosphoinositol headgroups with anion exchange HPLC. Values (dpm) are given as mean ± standard error of triplicate determinations.

	Untreated control	+Pertussis toxin		
		+Thrombin	Control	+Thrombin
PtdIns3P	702 ± 34	677 ± 42	784 ± 74	741 ± 78
PtdIns(3,4)P_2	110 ± 21	343 ± 33	124 ± 33	139 ± 39
PtdIns(3,4,5)P_3	121 ± 14	694 ± 12	91 ± 25	119 ± 29

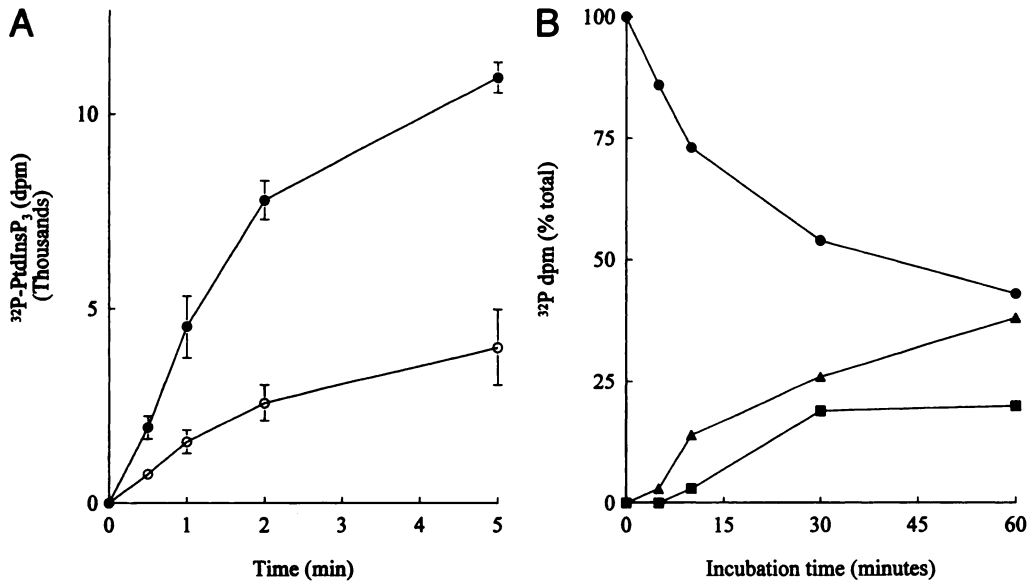

Fig. 2. Phosphoinositide-3-kinase and PtdIns(3,4,5)P_3-phosphatase activities in Ros 17/2.8 cell homogenates. A, Phosphoinositide-3-kinase activity was determined in Ros cell homogenates with PtdIns(4,5)P_2 as substrate and the methods described in the text. Formation of PtdIns(3,4,5)P_3 was determined in the presence (●) or absence (○) of 10 μM GTPγS. B, [3-^{32}P]PtdIns(3,4,5)P_3 was incubated with Ros cell homogenates, and radiolabeled products that were formed were analyzed as described in the text. ●, PtdIns(3,4,5)P_3; ▲, PtdIns(3,4)P_2; ■, PtdIns3P.

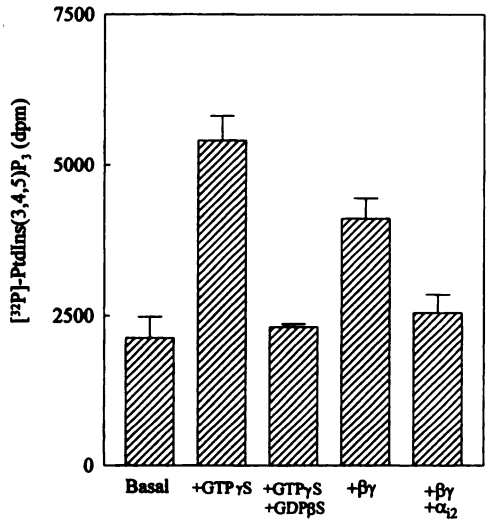

Fig. 3. Activation of phosphoinositide-3-kinase by guanine nucleotides and G protein βγ subunits in Ros 17/2.8 cell cytosol. Phosphoinositide-3-kinase assays were performed as described in the legend to Fig. 3. Assays contained the indicated guanine nucleotides and purified G protein subunits. The final concentrations were 10 μM GTPγs, 50 μM guanosine 5′-(β-thio)diphosphate, 1.6 μM βγ subunits, and 3.9 μM $G_{i\alpha 2}$.

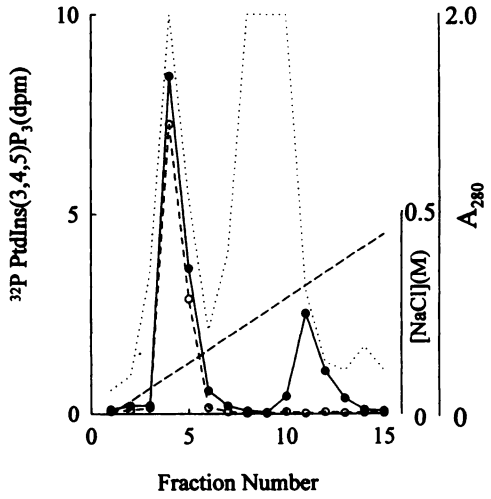

Fig. 4. Fractionation of Ros 17/2.8 cell phosphoinositide-3-kinase activities by anion exchange chromatography. A Ros cell cytosolic fraction was subjected to anion exchange chromatography on a Source 15Q column. Bound protein was eluted with a gradient of NaCl (*dashed line*) and monitored by absorbance at 280 nm (*dotted line*). Fractions were assayed for phosphoinositide-3-kinase activity with PtdIns(4,5)P_2 as substrate and the procedures described in the text in the absence (○) and presence (●) of 1 μM βγ subunits. Data are from one experiment.

units. These effects of βγ subunits were attenuated by preincubation with an excess (final concentration, 39 mM) of GDP-loaded α_{i2} before inclusion in the assay mixture (Fig. 5). Neither GTPγS at a concentration of 10 μM nor GTPγS-activated $G_{i\alpha 2}$ (final concentration, 390 nM) had an effect on the activity of the βγ subunit-responsive phosphoinositide-3-kinase (not shown).

Effect of βγ subunits on the p85α/p110β phosphoinositide-3-kinase heterodimer. Attempts to identify members of the p85/p110 forms of phosphoinositide-3-kinase in Ros 17/2.8 cell extracts or fractions from the anion exchange column by antisera raised against p110α, p110β, or p85α (provided by Dr. M. D. Waterfield, and prepared by ourselves or purchased commercially) were inconclusive. The βγ subunit-activated phosphoinositide-3-kinase activity of U937 cells appears to be distinct from the known phosphoinositide 3-kinase isoforms, whereas in human platelets a βγ subunit-activated phosphoinositide-3-kinase is apparently associated with the p85α regulatory subunit. We compared the effects of βγ subunits on the activities of the Ros 17/2.8 cell βγ subunit-sensitive phosphoinositide-3-kinase and on the activity of a widely expressed phosphoinositide-3-kinase catalytic subunit, p110β, either in monomer form or as a heterodimer associated with p85α. These proteins were expressed in sf9 cells with baculovirus vectors. The p85α/p110β heterodimer was purified to near homogeneity (Fig. 6A). The purified protein had a specific activity of approximately 150 nmol/min/mg with PtdIns(4,5)P_2 as substrate and was able to phosphorylate PtdIns, PtdIns4P, and PtdIns(4,5)P_2 *in vitro* with similar efficiencies under our standard assay conditions. Without coexpression of the p85α subunit, expression of p110β was considerably lower, the protein was much less

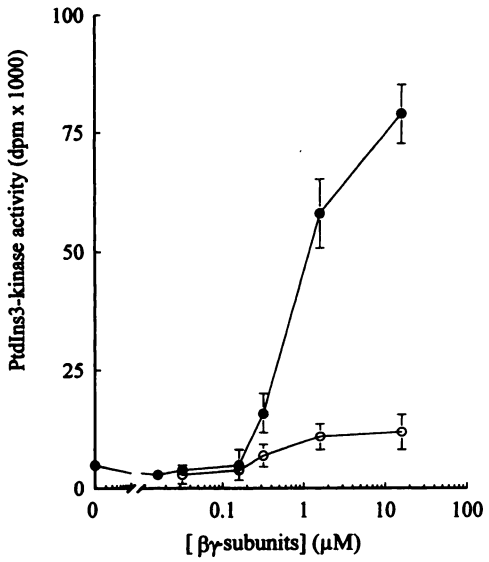

Fig. 5. Activation of partially purified Ros cell phosphoinositide-3-kinase by G protein βγ subunits. Ros cell βγ subunit-responsive phosphoinositide-3-kinase was isolated by anion exchange chromatography, and activity was determined as described in the text with PtdIns(4,5)P_2 as substrate. Assays contained 0.1% cholate and βγ subunits at the final concentrations indicated (●) or βγ subunits that had been preincubated with an excess of purified α_{i2} (○). Data are mean ± standard error of triplicate determinations.

stable during purification, and our final preparations contained a number of impurities, none of which reacted with a p85α antiserum on Western blotting (data not shown). Although the Ros 17/2.8 cell phosphoinositide-3-kinase was markedly activated by βγ subunits, the p85α/p110β phosphoinositide-3-kinase was insensitive to these activators (Fig. 6B). We consistently observed small (1.3-fold activation) effects of βγ subunits on monomeric p110β. We also evaluated the effects of the phosphoinositide-3-kinase inhibitor wortmannin on these phosphoinositide-3-kinase activities. When activated by βγ subunits, the Ros 17/2.8 cell βγ subunit-responsive phosphoinositide-3-kinase was inhibited by wortmannin (K_i, 50 nM), albeit with less sensitivity than the p85α/p110β heterodimer (approximate K_i, 0.6 nM; Fig. 6C).

Discussion

Activation of a variety of G protein-coupled receptors by ligand binding increases phosphoinositide-3-kinase activity in target cells. This type of regulation appears to be most prominent in cells of the hematopoietic lineage where receptors known to couple to the G_i class of pertussis toxin substrate G proteins can elicit rapid rises in levels of the 3-phosphorylated inositol lipids (9). Stimulation of 3-phosphorylated inositol lipid accumulation is clearly not a function of all G protein-coupled receptors, but the limited number of studies performed make it likely that the complement of G protein-coupled receptors and target cells capable of this mode of phosphoinositide-3-kinase regulation has not yet been defined (9, 38). Our results indicate that thrombin activates phosphoinositide-3-kinase in a fibroblastic cell line, Ros 17/2.8 cells. The maximal accumulation of PtdIns(3,4,5)P_3 observed (~2% of PtdIns(4,5)P_2) is clearly smaller than that reported for activation of phosphoinositide-3-kinase by G protein-coupled receptors in myeloid cells (7, 27, 28). Nevertheless, activation of phosphoinositide-3-kinase by thrombin in Ros 17/2.8 cells is rapid, and kinetic analysis of these changes in polyphosphoinositide levels suggests that, as in neutrophils, fibroblasts, and platelets, receptor-activated phosphoinositide-3-kinase is directed primarily against PtdIns(4,5)P_2 (7, 39, 40). The identification of an active PtdIns(3,4,5)$P_3$5-phosphatase in Ros 17/2.8 cell homogenates supports the idea that receptor promoted increases in PtdIns(3,4)P_2 and PtdIns3P levels are a consequence of rapid dephosphorylation of PtdIns(3,4,5)P_3 rather than direct phosphorylation of PtdIns and PtdIns4P, respectively, in these cells.

The protease-activated thrombin receptor is coupled to members of the G_i and G_q classes of heterotrimeric G proteins and thereby mediates inhibition of adenylylcyclase and activation of PLC in target cells (41). The observed susceptibility to inhibition by pertussis toxin implicates the G_i class of G proteins as mediators of the pathway by which the thrombin receptor activates phosphoinositide-3-kinase in intact Ros 17/2.8 cells. In broken cell assays, a nonhydrolysable guanine nucleotide trisphosphate analogue increased phosphoinositide-3-kinase activity measured with exogenously provided substrates with the specificity expected of a G protein-mediated process. Purified G protein βγ subunits also activated phosphoinositide-3-kinase in Ros 17/2.8 cell homogenates in a manner that was inhibited by preincubation with an excess of purified GDP-liganded α_{i2}. Two studies implicate the low-molecular-weight G proteins Rho and Ras as regulators of platelet phosphoinositide-3-kinase activity and of the p110α phosphoinositide-3-kinase catalytic subunit (27, 28). Ros 17/2.8 cells contain βγ subunit-sensitive and -insensitive phosphoinositide-3-kinase activities that can be resolved with anion exchange chromatography, and it is possible that the capacity of GTPγS to activate phosphoinositide-3-kinase in Ros 17/2.8 cell homogenates may be wholly or partly attributed to effects mediated by these small G proteins on one or both of these activities. Notwithstanding this possibility, taken together, our data suggest that release of G protein βγ subunits from the G_i class of G proteins may provide a biochemical basis for the ability of thrombin to activate phosphoinositide-3-kinase in intact Ros 17/2.8 cells.

G protein βγ subunits activate a range of effectors including the PLC-β isoenzymes, types II and IV adenylylcyclases (in the presence of $G_{s\alpha}$), the atrial inwardly rectifying potassium channel, and members of the G protein-coupled receptor kinase family (36–39; see Ref. 40 for a review). A partially purified preparation of the Ros 17/2.8 cell phosphoinositide-3-kinase was half-maximally activated by 0.6 μM βγ subunits. This concentration dependence is similar to that reported for the effects of βγ subunits on PLC-β isoenzymes and on the U937 cell phosphoinositide-3-kinase but is considerably higher than the concentrations of G protein α subunits required for activation of several structurally diverse effectors *in vitro* (29, 36–40). It is possible that this low potency is an artifact of the exogenous substrate assay system that was used, and it is easy to envisage problems of accessibility between a soluble phosphoinositide-3-kinase, its phospholipid substrate, and the G protein activator in the mixed phospholipid and detergent micelles used. In this context, it is important to note that the physical form and phospholipid composition of the substrate preparation employed can exert a profound effect on the catalytic and regulatory properties of many lipolytic and lipid-modifying enzymes. It

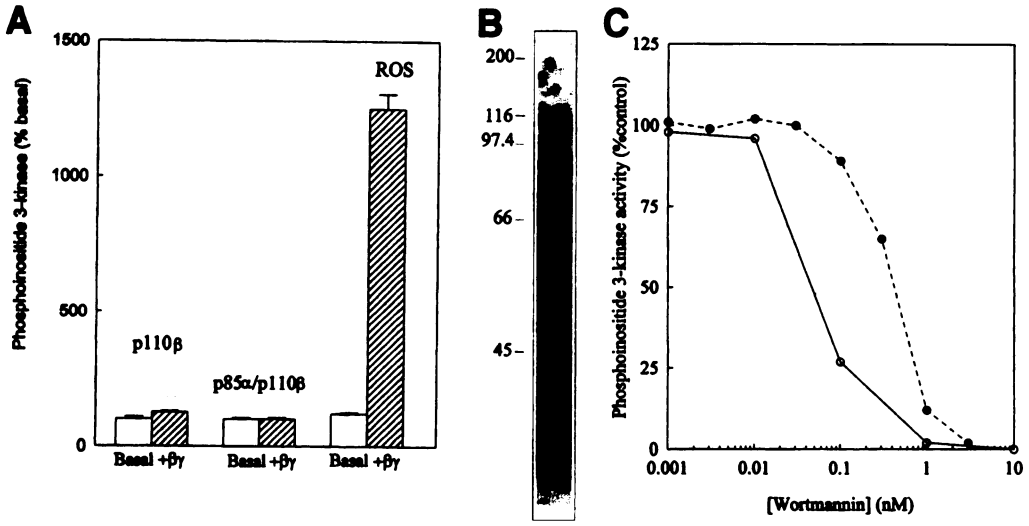

Fig. 6. Effects of βγ subunits and wortmannin on phosphoinositide-3-kinase and purified p85α/p110β. A, Purified p85α/p110β phosphoinositide-3-kinase heterodimer was analyzed with SDS-PAGE on a 7.5% gel. Proteins were visualized by staining with Coomassie blue. The molecular weights of marker proteins are indicated (kDa). B, p85α/p110β and the Ros 17/2.8 cell phosphoinositide-3-kinase were assayed with PtdIns(4,5)P_2 as substrate and the procedures described in the text. Activity was determined under either basal conditions or in the presence of 1.6 μM βγ subunits. Data are mean ± standard error of triplicate determinations. C, Wortmannin was included in the incubations and at the concentrations indicated, and phosphoinositide-3-kinase activity of the purified recombinant p85α/p110β heterodimer (○) or partially purified Ros 17/2.8 cell βγ subunit-responsive phosphoinositide-3-kinase (●) and was determined under standard conditions. The incubations with the Ros 17/2.8 cell phosphoinositide-3-kinase contained 1.6 μM βγ subunits. Data are mean values of triplicate determinations.

will be essential to investigate both the mechanism by which this soluble phosphoinositide-3-kinase catalyzes the phosphorylation of membrane-localized phospholipid substrates and the process by which G protein βγ subunits increase its activity. It is also noteworthy that the preparations of bovine brain βγ subunits used for these experiments are mixtures of dimers assembled from distinct β and γ subunit gene products, and it is plausible that some subset of these may be more potent activators of the βγ subunit-responsive phosphoinositide-3-kinase. This does not appear to be the case for other βγ subunit-activated effectors. With one exception (the $\beta_1\gamma_1$ dimer), a variety of recombinantly assembled βγ subunit dimers are equipotent activators of types II and IV adenylyl-cyclases, PLC-β isoenzymes, and the atrial potassium channel. The reasons underlying this apparent disparity in concentration dependence for activation of effectors by G protein α and βγ subunits is still a matter of debate. It is also unclear how the receptor-selective activation of G protein βγ subunit-responsive effectors observed *in vivo* is achieved in systems expressing multiple G protein-coupled receptors (40).

The identity of the βγ subunit-activated phosphoinositide-3-kinase isoform is unknown. Resolution of this issue and demonstration of direct activation by βγ subunits will clearly require purification and sequence analysis of this enzyme. In U937 cells, the βγ subunit-responsive phosphoinositide-3-kinase is chromatographically and functionally distinct from the known heterodimeric phosphoinositide-3-kinase isoenzymes. The acute βγ subunit responsiveness of the Ros 17/2.8 cell phosphoinositide-3-kinase and its behavior during anion exchange chromatography suggest that it is related to the U937 cell enzyme. A βγ subunit-stimulated phosphoinositide-3-kinase activity in human platelet cytosol could be depleted by strategies expected to remove phosphoinositide-3-kinase catalysis associated with a p85 regulatory subunit. p110β is a widely expressed form of the heterodimeric phosphoinositide-3-kinase catalytic subunit. We expressed p110β in sf9 cells with baculovirus vectors and purified this protein in either monomer form or as a heterodimer associated with the p85α regulatory subunit. The heterodimeric preparations of this enzyme were insensitive to activation by G protein βγ subunits, and monomeric p110β was very weakly activated by βγ subunits.

Finally, the role of receptor activation of phosphoinositide-3-kinase in cellular responses to stimulation by diverse agonists is unknown. Obviously, the key question in this field concerns the precise physiological function of this enzyme and delineation of the presumed biochemical targets of its phospholipid products. Building a detailed understanding of the proteins responsible for the cellular pathway of synthesis and degradation of the 3-phosphorylated inositol lipids is central to answering this question. The description of a pathway for phosphoinositide-3-kinase activation that is apparently mediated by G protein βγ subunits adds to the potential diversity of receptor ligands that can activate this putative signaling pathway.

Acknowledgments

We thank Drs. Craig C. Malbon and David L. Williams for their assistance and Drs. Patrick Hu and Joseph Schlessinger for their generous provision of the p85α and p110β cDNAs.

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