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# G-protein-coupled Receptor Agonists Activate Endogenous Phospholipase Cε and Phospholipase Cβ3 in a Temporally Distinct Manner*

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## Abstract

Phospholipase Cε (PLCε) is one of the newest members of the phosphatidylinositol-specific phospholipase C (PLC) family. Previous studies have suggested that G-protein-coupled receptors (GPCRs) stimulate phosphoinositide (PI) hydrolysis by activating PLCβ isoforms through $G_q$ family G proteins and Gβγ subunits. Using RNA interference to knock down PLC isoforms, we demonstrate that the GPCR agonists endothelin (ET-1), lysophosphatidic acid (LPA), and thrombin, acting through endogenous receptors, couple to both endogenous PLCε and the PLCβ isoform, PLCβ3, in Rat-1 fibroblasts. Examination of the temporal activation of these PLC isoforms, however, reveals agonist- and isoform-specific profiles. PLCβ3 is activated acutely within the first minute of ET-1, LPA, or thrombin stimulation but does not contribute to sustained PI hydrolysis induced by LPA or thrombin and accounts for only part of ET-1 sustained stimulation. PLCε, on the other hand, predominantly accounts for sustained PI hydrolysis. Consistent with this observation, reconstitution of PLCε in knockdown cells dose-dependently increases sustained, but not acute, agonist-stimulated PI hydrolysis. Furthermore, combined knockdown of both PLCε and PLCβ3 additively inhibits PI hydrolysis, suggesting independent regulation of each isoform. Importantly, ubiquitination of inositol 1,4,5-trisphosphate receptors correlates with sustained, but not acute, activation of PLCε or PLCβ3. In conclusion, GPCR agonists ET-1, LPA, and thrombin activate endogenous PLCε and PLCβ3 in Rat-1 fibroblasts. Activation of these PLC isoforms displays agonist-specific temporal profiles; however, PLCβ3 is predominantly involved in acute and PLCε in sustained PI hydrolysis.

The phosphatidylinositol-specific phospholipase C (PLC)[2] family is a group of critical cellular signaling enzymes that hydrolyze phosphatidylinositol 4,5-bisphosphate (PI) to generate inositol 1,4,5-trisphosphate($IP_3$) and diacylglycerol, which increase the intracellular free $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) and activate protein kinase C, respectively (1,2). Eleven isoforms of PLC, representing five distinct, differentially regulated classes, have been identified: PLCβ1 to β4; PLCγ1 and γ2; PLCδ1, δ3, and δ4; and PLCε and PLCζ. PLCβ is regulated by

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2The abbreviations used are: PLC, phosphoinositide-specific phospholipase C; LPA, lysophosphatidic acid; ET-1, endothelin; siRNA, short interfering RNA; FBS, fetal bovine serum; PI, phosphatidylinositol 4,5-bisphosphate; IP, inositol phosphate; $IP_3$, inositol 1,4,5-trisphosphate; $IP_3R$, inositol 1,4,5-trisphosphate receptor; S1P, sphingosine 1-phosphate; GPCR, G-protein-coupled receptor.

G-protein-coupled receptor (GPCR) activation of heterotrimeric $G_q$ family G-proteins and Gβγ subunits. PLCγ is regulated by tyrosine phosphorylation by receptor tyrosine kinases (*e.g.* epidermal growth factor and platelet-derived growth factor) and nonreceptor tyrosine kinases (*e.g.* Src) activated by immunoglobulin and cytokines. Regulation of PLCδ is less well understood but is probably regulated by changes in $[Ca^{2+}]_i$, possibly downstream from activation of other PLC isoforms, and by high molecular weight G-protein, $G_h$. PLCζ is also regulated by $[Ca^{2+}]_i$ (3).

PLCε was discovered only recently and is the largest member of the PLC family (4–7). PLCε is regulated by the monomeric Ras (4,8,9) and Rho (9,10) families, the heterotrimeric G12 family (5,9), and Gβγ subunits (11). Consistent with its diverse regulation by G-proteins, studies utilizing overexpressed PLCε suggest that this isoform is regulated by receptor tyrosine kinases and GPCRs. Receptor tyrosine kinase agonists, epidermal growth factor (9), and platelet-derived growth factor (8) have been shown to stimulate PLCε through Ras and Rap. GPCR-mediated activation of PLCε can be subdivided into two groups. One group, $\beta_2$ adrenergic, prostanoid, and muscarinic $M_3$ receptor agonists, has been proposed to stimulate PLCε through Rap2B mediated by cAMP-dependent activation of the Rap GTP exchange factor EPAC (12,13). The second group, lysophosphatidic acid (LPA), sphingosine 1-phosphate (S1P), and thrombin receptor agonists, activate PLCε through $G\alpha_{12/13}$ and Rap (9).

Accumulating evidence suggests that activation of PLC by hormones is more complex than a simple linear activation from receptor to one PLC isoform (1,2). For example, GPCR receptor activation of PLCβ isoforms may lead to activation of PLCδ isoforms through increased levels of $[Ca^{2+}]_i$ (14,15). Alternatively, $\alpha_{1B}$-adrenergic receptor activation has been shown to stimulate PLCβ through $G\alpha_{q/11}$ and to a lesser extent PLCδ1 through $G_h$ (16). GPCRs have been shown to transactivate receptor tyrosine kinases (17,18) with potential activation of PLCβ and PLCγ, respectively. In addition, angiotensin II activation of the GPCR $AT_1$ has been proposed to activate PLCγ1 (19) and possibly temporally activate PLCβ1 (<30 s) followed by PLCγ1 (>30 s) (20). Furthermore, recent studies suggest that PLCβ2 and PLCδ1 exist as a heterodimer that sequesters and inhibits PLCδ1 activity (21). Upon Gβγ activation of PLCβ2, PLCδ1 is released, allowing the enzyme to hydrolyze its substrate. Thus, receptor activation of cellular PLC activity is probably the composite of a complex signaling network that involves the activation of multiple PLC isoforms. Since similar receptor agonists have been postulated to stimulate PLCε and the classic isoforms, PLCβ and PLCγ, it is possible that novel signaling pathways, that redefine current PLC dogma, participate in this network.

In the present studies, we used RNA interference to elucidate the physiologic regulation of PLCε and the PLC network regulated by GPCR agonists in Rat-1 fibroblasts. Rat-1 fibroblasts are a classic model for examining hormonal regulation of downstream effectors, and we show that these cells are easily manipulated by RNA interference using retrovirally transduced short interfering hairpin RNAs to generate stable and almost complete knockdown of signaling proteins. The GPCR agonists, endothelin, LPA, and thrombin, have been shown to stimulate PLC in Rat-1 fibroblasts through endogenous receptors (22–24), and previous studies suggest that these agonists couple to PLCβ isoforms (25–27). Here, we demonstrate for the first time that these GPCR agonists activate both endogenous PLCε and PLCβ3 through endogenous receptors in Rat-1 fibroblasts. Activation of these isoforms, however, exhibits distinct agonist- and isoform-specific temporal profiles whereby PLCβ3 predominantly participates in acute and PLCε sustained PLC activity. In addition, sustained activation of PLCε is functionally correlated with $IP_3$ receptor ubiquitination, a protective process associated with sustained activation of PLC.

# EXPERIMENTAL PROCEDURES

## Materials

PLCβ1 and PLCγ1 antibodies were from Upstate Biotechnologies, Inc. (Lake Placid, NY). PLCβ2, PLCβ3, PLCβ4, PLCγ2, PLCδ1, PLCδ2, and PLCδ3 antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). LPA was from Sigma, α-thrombin was from Hematologic Technologies, Inc. (Essex Junction, VT), and endothelin-1 was from Calbiochem. Anti-ubiquitin, FK2, was from Biomol International L.P. (Plymouth Meeting, PA). The PLCε antibody used in these studies was a rabbit polyclonal antibody generated from the RA1 domain. The type 1 $IP_3$ receptor antibody was a rabbit polyclonal antibody generated to a C-terminal peptide (28).

## Tissue Culture

Rat-1 fibroblasts and all HEK293 cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. For reconstitution studies, Rat-1 clonal cell lines were seeded into 24-well dishes at $8 \times 10^4$ 6 h prior to adenoviral transduction. Medium was then removed, and adenoviruses at the appropriate concentrations were applied in 250 μl of complete Dulbecco's modified Eagle's medium for 24 h. Cells were then labeled for 18–24 h in inositol-free, serum-free Dulbecco's modified Eagle's medium. For all other PLC assays, Rat-1 cells were seeded into 24-well dishes at $4 \times 10^4$, grown overnight, and labeled the following day for 18–24 h in inositol-free, serum-free Dulbecco's modified Eagle's medium.

## Plasmid Constructs

Short interfering hairpin RNAs (siRNAs) were designed for PLCε and PLCβ3 targeting rat (r), mouse (m), or human (h) sequences: siRNAPLCε#1, GCCGGCATTCCTAAGACAC (r); siRNAPLCε#3, GGCACCAAGGCAAAGCAGC (rmh); siRNAPLCε#5, GCCAAATATTCCTACAGCA (rmh); siRNAPLCβ3#2, CATGGAGGTGGACACACTG (rmh); siRNAPLCβ3#3, GGTCTGGTCAGAGGAGTTG (rm); siRNAPLCβ3#5, GCTTCTGACTACATCCCAG (rm); siRNARan#5, GTAACATCGCCACGCGTCA (scrambled siRNAPLCε#1); siRNARan#6, CAGACACCGCAGCAGGAGA (scrambled siRNAPLCε#3); siRNARan#3, ACTGTCACAAGTACCTACA (scrambled siRNAPLCε#5).

Sense (5′-gatcccc(siRNA)ttcaagaga(antisense siRNA)tttttggaaa and antisense (5′-agcttttccaaaaa(siRNA)tctcttgaa(antisense siRNA)ggg) oligonucleotides incorporating these siRNAs were synthesized (Sigma) and cloned into pSUPER and/or pSUPER-Retro (29), generous gifts of Dr. R. Agami.

Silent mutations were introduced into rat PLCε, pcDNA-PLCε-FLAG, to generate pcDNA-PLCεSI#5. Mutations were introduced by site-directed mutagenesis using the sense primer, 5′-CAAAGCCAAATAcagtTACAGCATCC, to mutate TTCC to cagt (shown in lowercase type) (Quick Mutagenesis Kit, Stratagene, La Jolla, CA). The mutated region was sequenced and subcloned into the original plasmid.

The cDNA for PLCεSI#5 was subcloned into the pShuttle-CMV vector, and recombinant adenovirus was generated following the manufacturer's protocol for the Stratagene AdEasy Adenoviral Vector System (Stratagene).

Similarly, the lipase-inactive mutant (5), H1433L PLCε, was created by site-directed mutagenesis. The region encompassing this mutation was subcloned into PLCεSI#5, which was subsequently subcloned into pShuttle-CMV, and recombinant adenovirus, Adsi#5/

H1433L PLCε, was produced. All constructs were sequenced (DNA Sequencing and Synthesis Facility, Iowa State University, Ames, IA).

### Stable Retroviral Knockdown

To generate retroviruses, HEK293T cells were seeded into T75 flasks at $8 \times 10^6$ the previous day. Cells were transfected with pSUPER-Retro siRNA constructs, pVPackECO, and pVPackGP plasmids by $Ca_2PO_4$ precipitation. Viruses were harvested after 48 h by filtering medium and quick freezing on dry ice. Stocks were stored at −80 °C. Viruses were titered by transducing Rat-1 cells with serial dilutions, killing untransduced cells with 3 μg/ml puromycin, and counting remaining colonies.

Rat-1 fibroblasts were seeded in 12-well plates at $4 \times 10^4$. The following day, the conditioned medium was aspirated off, and 1 ml of viral supernatant and 8 μg/ml Polybrene were applied to each well for 6–8 h. The virus was replaced with complete medium after incubation. Cells were expanded to T75 flasks the next day, and 3 μg/ml puromycin was added to medium 48 h after transduction.

### Adenovirus Production

Generation of primary adenovirus was performed according to the AdEasy Adenoviral Vector System (Stratagene) protocol, except that the transfected cultures were incubated for 18 days or until a visible cytopathic effect was observed. Viruses were amplified by several rounds of infection in AD293 cells. Viral titers were calculated by an immunoreactivity spot assay (30).

### Phospholipase C Assay

The PLC assay was performed under two different conditions, 20 and 100 mM LiCl. Experiments using 20 mM $Li^+$ were performed as previously described (4,9). Basically, agonists were added to the conditioned labeling medium brought to a final concentration of 20 mM LiCl, and the cells were incubated for 60 min unless indicated otherwise. Agonists were dissolved as follows: LPA, 0.1% bovine serum albumin in phosphate-buffered saline; thrombin, 0.1% PEG, 1 mg/ml bovine serum albumin in phosphate-buffered saline; and endothelin-1 (ET-1), double-distilled $H_2O$.

To increase trapping of inositol phosphates (IPs), 100 mM LiCl has been used in Rat-1 cells (22,31). For experiments performed in 100 mM LiCl (Figs. 3–7), conditioned labeling medium was replaced with Krebs Buffer 1 (4.7 mM KCl, 1.2 mM $KH_2PO_4$, 25 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 10 mM HEPES, 1.3 mM $CaCl_2$, 118 mM NaCl, pH 7.4, aerated with 5% $CO_2$/95% $O_2$ for 10 min), and the Rat-1 cells were allowed to equilibrate for 60 min in a $CO_2$ incubator at 37 °C. Prior to the addition of agonists, cells were preincubated in Krebs Buffer 2 (4.7 mM KCl, 1.2 mM $KH_2PO_4$, 25 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 10 mM HEPES, 1.3 mM $CaCl_2$, 18 mM NaCl, 100 mM LiCl, pH 7.4, aerated with 5% $CO_2$/95% $O_2$ for 10 min) for 10 min. The reaction was stopped by aspirating off the incubation medium and adding 375 μl of ice-cold 50 mM formic acid. After 30 min of incubation on ice, samples were removed, neutralized with 125 μl of 150 mM ammonium hydroxide, and diluted with 5 ml of ice-cold water. Total inositol phosphates were separated by column chromatography using AG 1-X8 200–400 mesh, formate form, and quantitated by liquid scintillation counting. Protein determination (Bio-Rad DC Protein Assay Kit) and expression by Western blot analysis were determined in parallel wells carried through the PLC assay but without radioactivity. Steady state labeling of phosphatidylinositol was not significantly different between conditions.

### $IP_3$ Mass Assay

Change in $IP_3$ mass was determined as previously described (32). Rat-1 cells were seeded at a density of $1.9 \times 10^5$/6-well plate and then incubated in a 5% $CO_2$ incubator at 37 °C for 24 h and then serum-starved for an additional 24 h. Incubations were initiated by the addition of agonist and were terminated by removing medium and adding 250 μl of ice-cold 0.5 M trichloroacetic acid. After 2 h at 4 °C, the 250 μl was removed and mixed with 50 μl of 10 mM EDTA and 250 μl of Freon/*N*-trioctylamine (1:1), vortexed, incubated at 4 °C for 15 min, and centrifuged (16,000 × *g* for 5 min at 4 °C). A portion of the aqueous phase (150 μl) was then removed and neutralized with 75 μl of 25 mM $NaHCO_3$, and $IP_3$ concentration was determined with a radioreceptor assay as described (33).

### $IP_3$ Receptor Ubiquitination

$IP_3$ receptor ubiquitination was measured essentially as previously described (34). Rat-1 cells were grown to near confluence in 15-cm diameter dishes and were serum-starved for 15 h. For reconstitution studies, cells were transduced 6 h after seeding for 24 h with the indicated adenoviruses prior to serum starvation. Cells were then preincubated with the proteasome inhibitor bortezomib and exposed to stimuli. After removing culture medium, cells were solubilized by adding lysis buffer (50 mM Tris-base, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 10 μM pepstatin, 0.2 μM soybean trypsin inhibitor, 1 mM dithiothreitol, pH 8) directly to the monolayers, followed by vigorous scraping. After 30 min at 4 °C, lysates were clarified by centrifugation (16,000 × *g* for 10 min at 4 °C), and $IP_3R1$ was immunoprecipitated by incubating overnight with anti-$IP_3R1$ and Protein A-Sepharose CL-4B. Immunocomplexes were washed thoroughly with lysis buffer, resuspended in gel loading buffer, electrophoresed, and probed in immunoblots with anti-ubiquitin and anti-$IP_3R1$.

### Calculations and Statistical Analysis

Percentage change induced by knockdown of PLCε or PLCβ3 compared with random control cell lines (percentage change of siRNARan) was determined to summarize effects of PLC isoform knockdown. To calculate this value, basal levels (activity without added GPCR agonist) were subtracted from stimulated levels. Activity in PLCε or PLCβ3 cell lines was then divided by the activity in random siRNA-treated control cells, and from this value percentage change was calculated.

Paired or unpaired Student's *t* test, Tukey-Kramer multiple comparisons test, and one sample *t* test were performed where appropriate. A value of $p < 0.05$ was considered significant.

## RESULTS

### Serum and G-protein-coupled Receptor Agonists Are Coupled to Endogenous PLCε in Rat-1 Fibroblasts

To find a cell line in which GPCR agonists couple to endogenous PLCε, various cell lines were screened by Western blotting, and Rat-1 fibroblasts were determined to express high levels of PLCε. Rat-1 fibroblasts are a classic cell line to study hormonal regulation of PLC, and the GPCR agonists endothelin (ET-1) and thrombin have been shown to stimulate PLC in this cell line (22–24). To determine whether these agonists activate PLCε, RNA interference was used to knock down endogenous PLCε. Eight siRNAs targeting PLCε were generated, which knocked down PLCε with different efficiencies. Of these, three (siRNAPLCε#1, siRNAPLCε#3, and siRNAPLCε#5) were used to generate stable cell populations that knock down PLCε in Rat-1 fibroblasts by 53–97% (Fig. 1*B*). Random sequences of these siRNAs were used as controls. Knockdown of PLCε did not affect the expression of the other PLC

isoforms present in this cell line, PLCβ3, PLCγ1, or PLCδ1 (Fig. 1*B*); expression of G-proteins, $G_{12}$, $G_{13}$, Rho, Ras, or Rap (data not shown); or cell growth (data not shown).

Fig. 1*A* shows the effect of knocking down PLCε on fetal bovine serum (FBS), ET-1, and thrombin-stimulated PLC activity. With increasing knockdown, agonist-stimulated PLC activity was increasingly inhibited compared with three different random siRNA control cell populations. The two most effective siRNAs, siRNAPLCε#1 and siRNAPLCε#5, significantly inhibited FBS-stimulated PI hydrolysis by 39 and 74%, respectively, ET-1 by 30 and 69%, and thrombin by 69 and 94%. Similar results were obtained in an independently generated set of stable cell lines (data not shown). Significant inhibition by at least two distinct siRNAs suggests that the observed inhibition was not due to off-target effects. Moreover, uncoupling was not due to siRNA-induced nonspecific activation of interferon, since an increase in STAT-1 phosphorylation was not observed in these stable cell populations (data not shown). In addition, the comparable agonist response in each of the three random cell lines (Fig. 1*A*) suggests that the inhibition was unlikely to be due to cell population variability induced by retroviral transduction and stable cell population selection. Taken together, these studies demonstrate that FBS, ET-1, and thrombin are coupled to PLCε in Rat-1 fibroblasts.

## G-protein-coupled Receptor Agonists Are Coupled to PLCβ3 in Rat-1 Fibroblasts

Classically, endothelin and thrombin are thought to activate PLCβ isoforms through $G_q$ family G-proteins and Gβγ subunits (25,27). We found that Rat-1 fibroblasts express high levels of PLCβ3 (Fig. 2*B*), but the other three PLCβ isoforms, PLCβ1, -2, and -4, were not detected (data not shown). Because knockdown of PLCε did not completely inhibit stimulation of PLC by these agonists, PLCβ3 was knocked down to determine whether these agonists couple to this PLC isoform. Seven siRNAs were screened, and the three most effective, siRNAPLCβ3#2, siRNAPLCβ3#3, and siRNAPLCβ3#5, were used to generate stable cell populations. These siRNAs knocked down PLCβ3 by 88–96% (Fig. 2*B*).

Fig. 2*A* shows that knocking down PLCβ3 with each of these siRNAs significantly inhibited ET-1- and thrombin-stimulated PLC activity. The two most effective siRNAs, siRNAPLCβ3#3 and siRNAPLCβ3#5, significantly inhibited ET-1-stimulated PI hydrolysis by 44 and 39%, respectively, and thrombin by 66 and 69%. FBS stimulation, on the other hand, was less affected by knockdown of PLCβ3 and was only significantly inhibited by 32% with siRNAPLCβ3#3 but not by siRNAPLCβ3#5. Thus, in addition to being coupled to PLCε, ET-1 and thrombin are also coupled to PLCβ3 in Rat-1 fibroblasts, consistent with previous studies that have suggested that these $G_q$ and $G_i$ family-coupled GPCR agonists activate PLCβ isoforms in these cells. FBS, however, appears to predominantly regulate PLCε.

## Distinct Temporal Activation of PLCβ3 and PLCε

Because knockdown experiments demonstrated that ET-1 and thrombin are coupled to both PLCβ3 and PLCε, the kinetics of the hormonal regulation of each isoform was examined to determine its unique contribution to total cellular PLC activity. In addition, LPA has been shown to stimulate PLC activity in Rat-1 fibroblasts (35), and its temporal regulation of PLCε and PLCβ3 was also determined. For these experiments, a set of stable cell populations was generated with the most effective PLC-specific siRNAs, siRNAPLCε#5 and siRNAPLCβ3#3, and a random siRNA control. To increase knockdown, cells were transduced twice with retrovirus, which increased knockdown of each PLC isoform to greater than 99% compared with a random siRNA control (Fig. 3*D*). In addition, initial studies demonstrated that 20 $m_M$ $Li^+$ was not sufficient to block degradation of sustained IP generation (data not shown). Lithium is a noncompetitive inhibitor of inositol monophosphatase, and therefore high concentrations are required to inhibit degradation of low concentrations of inositol phosphates

(IPs). Thus, the concentration of $Li^+$ was increased to 100 mM as previously described in these cells (22,31).

Fig. 3*A* shows the temporal effect of knocking down PLCε or PLCβ3 on FBS-, ET-1-, LPA-, and thrombin-stimulated total IP accumulation compared with a random control. FBS and each agonist effectively increased total inositol phosphate accumulation. The stimulation by ET-1, LPA, and thrombin was biphasic with an initial acute response (1 min) followed by a sustained increase (3–60 min). FBS stimulation was monophasic with no significant acute response. As shown above (Figs. 1 and 2), knockdown of PLCε or PLCβ3 inhibited serum-, ET-1-, and thrombin-stimulated PLC activity. In addition, LPA stimulation was also inhibited. Fig. 3, however, shows that knockdown of PLCε or PLCβ3 had differential effects on the temporal stimulation induced by these agonists.

This was most apparent for LPA and thrombin, which showed a crossover at 3 min in the temporal activation profiles of the PLCε or PLCβ3 knockdown cell lines (Fig. 3*A*). PLCε knockdown had no significant effect on the acute stimulation by LPA or thrombin (Fig. 3*B*); however, it markedly inhibited the sustained response to these agonists by ~80% at 60 min (Fig. 3*C*). When trapped IPs at 1 min are subtracted from later time points, knockdown of PLCε effectively abolished the sustained stimulatory response. Conversely, PLCβ3 knockdown inhibited LPA and thrombin acutely by ~50% but had no effect on sustained stimulation. Thus, LPA and thrombin predominantly activate PLCβ3 during acute stimulation and PLCε during sustained stimulation.

Similarly, acute stimulation of PI hydrolysis by ET-1 was significantly inhibited by knockdown of PLCβ3 and the sustained stimulation by knockdown of PLCε. However, differences were noted. Whereas knockdown of PLCβ3 inhibited LPA or thrombin stimulation by ~50%, ET-1 was only inhibited by 20% (Fig. 3*B*). Furthermore, in contrast to LPA and thrombin, knockdown of PLCε only inhibited the sustained response by 44% and PLCβ3 inhibited the sustained response by 27% (Fig. 3*C*). Thus, whereas the activation profiles of these PLC isoforms by ET-1 are similar to LPA and thrombin, agonist-dependent differences exist.

### Effect of PLCε and PLCβ3 Knockdown on Acute PI Hydrolysis

Since there appeared to be a differential effect of PLCε or PLCβ3 knockdown on acute stimulation of PI hydrolysis, a shorter time course was performed to better define the boundaries of the acute stimulatory component. In addition, changes in total IP accumulation were correlated with $IP_3$ generation. Fig. 4 shows the effect of knocking down PLCε or PLCβ3 on GPCR agonist-stimulated total IP accumulation and $IP_3$ mass. ET-1, LPA, and thrombin effectively stimulated total IP accumulation acutely, and the responses were near maximal by 30 s (Fig. 4*A*). Knockdown of PLCβ3, but not PLCε, inhibited this acute stimulation (Fig. 4*A*) by 30–60% (Fig. 4*C*).

Changes in total IP accumulation correlated with changes in $IP_3$ mass. ET-1, LPA, and thrombin markedly stimulated $IP_3$ generation, which peaked at 15 s and decreased to levels modestly above basal levels by 90 s (Fig. 4*B*). Knockdown of PLCβ3, but not PLCε, inhibited this $IP_3$ generation by 30–50% (Fig. 4*D*), comparable with inhibition of total inositol phosphate accumulation (Fig. 4*C*). These studies confirm the selective role of PLCβ3 during the acute GPCR stimulation of PI hydrolysis.

Because sustained $IP_3$ levels are low, quantitation of the effects of PLC isoform knockdown by measuring $IP_3$ is not as sensitive as measuring total IP accumulation in the presence of $Li^+$. Therefore, changes in the mass of $IP_3$ at sustained time points were not determined except for the effect of PLCε knockdown on ET-1-stimulated PI hydrolysis, which appeared elevated at 90 s (Fig. 4*B*). In this set of experiments, PLCε knockdown significantly inhibited the

sustained ET-1 stimulation of $IP_3$ measured at 20 min by 90 ± 8% from 75 pmol/mg protein to 9 ± 6 ($p < 0.02$; $n = 3$), consistent with total IP measurements and a role for PLCε in sustained $IP_3$ formation.

## Dose- and Agonist-dependent, Differential Effects of PLCε and PLCβ3 Knockdown on Acute and Sustained PLC Activity

To further assess the effects of PLCε and PLCβ3 knockdown on agonist-stimulated acute and sustained PLC activity and to determine whether lower agonist concentrations would differentially affect coupling to either isoform, a set of experiments was performed with increasing concentrations of agonist, and total IP accumulation was determined. Acute stimulation was determined by measuring IP accumulation at 1 min after agonist addition. Cells were preincubated with $Li^+$ for 10 min. For sustained stimulation, agonist was added similarly, but $Li^+$ was not added until 3 min after agonist addition, and the experiment was terminated at 60 min. This effectively measures sustained PLC activity from greater than 3 min (time for $Li^+$ to block IP degradation) to 60 min; IPs formed at times less than 3 min are not trapped, because $Li^+$ is not present.

Fig. 5 shows dose responses to ET-1, LPA, and thrombin and the effect of knocking down PLCε or PLCβ3 on acute (1-min) and sustained (3–60-min) IP accumulation. ET-1 stimulated acute PLC activity with an $EC_{50}$ of ~20 $n_M$ (Fig. 5*A*) and sustained activity with an $EC_{50}$ of 0.4 $n_M$ (Fig. 5*B*), ~50-fold more potent than acute. At 1 $n_M$, sustained stimulation was near maximal, whereas there was almost no acute stimulation. At all stimulatory doses, there was no shift in contribution of PLCε or PLCβ3 to total PLC activity; PLCβ3 knockdown inhibited acute and PLCε and PLCβ3 knockdown both inhibited sustained PI hydrolysis.

Similarly, thrombin stimulated acute PI hydrolysis with an $EC_{50}$ of 200 $p_M$ (Fig. 5*A*) and sustained >50-fold more potently with an $EC_{50}$ of 3 $p_M$ (Fig. 5*B*). At 10 $p_M$, sustained PLC activity was maximal with very little acute stimulation. At all stimulatory concentrations, acute stimulation was inhibited by PLCβ3 knockdown but not PLCε knockdown (Fig. 5*A*), in contrast to sustained stimulation, which was almost completely abrogated with PLCε knockdown and not affected by PLCβ3 knockdown (Fig. 5*B*).

The dose response to LPA was somewhat more complicated. Both acute and sustained stimulation appeared biphasic with an initial $EC_{50}$ of 30–100 $n_M$ for acute (Fig. 5*A*) and sustained stimulation (Fig. 5*B*) followed by a second phase with an $EC_{50}$ of 0.6–3 $\mu_M$. The $EC_{50}$ of the first phase is comparable with known LPA receptors, whereas the second is possibly mediated by a low affinity LPA receptor that appears important for mitogenesis but has yet to be identified (26). Acute stimulation was inhibited by ~50% at all stimulatory concentrations by knockdown of PLCβ3 but not affected by knockdown of PLCε (Fig. 5*A*). In contrast, knockdown of PLCβ3 had no effect on sustained stimulation. The first phase of the sustained stimulation, however, was entirely dependent on PLCε, since knockdown of PLCε completely inhibited PI hydrolysis (Fig. 5*B*). The second sustained phase, on the other hand, was accompanied by a component that was not inhibited by PLCε or PLCβ3 knockdown, suggesting that another PLC isoform is activated at high LPA concentrations.

## Reconstitution and Overexpression of PLCε

To further examine the role of PLCε in sustained PI hydrolysis, a PLCε construct, AdPLCε#5, was generated with silent mutations in the region targeted by siRNAPLCε#5 to prevent siRNA-mediated degradation. Transduction of Rat-1 cells reduced PLC activity for unknown reasons, but the agonist stimulatory profiles remained intact. Fig. 5, *A* and *C*, show that when PLCε is reconstituted to levels present in random siRNA-treated cells (Fig. 5, *B* and *E*), the profiles of LPA (Fig. 5*A*) and thrombin (Fig. 5*C*) stimulation of sustained IP accumulation are restored.

These studies confirm that the observed effects of knocking down PLCε in the siRNAPLCε#5 cell line on the sustained agonist-stimulatory profiles are specific and not due to off-target effects of the siRNA.

In addition, Fig. 5*D* shows that overexpressing PLCε selectively enhances the sustained increase in IP accumulation stimulated by thrombin. In this set of experiments, a random cell line was transduced with LacZ and a PLCε knockdown cell line with increasing amounts of adenovirus expressing PLCε (Fig. 5*E*). Overexpressing PLCε slightly increased basal, unstimulated IP accumulation from 13.4 ± 1.0 cpm/μg protein in AdLacZ-treated cells to 17.4 ± 3.6 and 21.8 ± 0.4 for cells moderately (multiplicity of infection 20), and highly (multiplicity of infection 60) overexpressing PLCε, respectively. However, the -fold stimulation acutely at 1 min was not different. On the other hand, the sustained PI hydrolysis was markedly potentiated. At 30 and 60 min, IP accumulation in the moderately overexpressing cells was 1.6- and 2.2-fold above the control stimulation (-fold stimulation of the PLCε-over-expressing cell line/-fold stimulation of the Ran#6 LacZ-expressing cell line), respectively, and in the highly expressing cells it was 3.6- and 5.1-fold, respectively. Thus, overexpressing PLCε has no effect on acute thrombin stimulation but markedly potentiates sustained stimulation, consistent with the knockdown experiments.

## Double Knockdown of PLCε and PLCβ3

To determine whether the activations of PLCε and PLCβ3 are independent, both PLCε and PLCβ3 were knocked down, and agonist-stimulated PLC activity was compared with a random siRNA-treated cell line. A set of stable cell populations was generated in which PLCε and PLCβ3 were knocked down >99 and 92%, respectively, compared with a random control (Fig. 6*B*). Fig. 6*A* shows the stimulatory response to ET-1, LPA, and thrombin in these cell lines. In addition, the stimulatory response observed in single knockdown cell lines is shown. For each agonist, IP accumulation was inhibited to a similar extent as the additive knockdown of the single knockdown cell lines. This would suggest that PLCε and PLCβ3 are regulated independently by these GPCR agonists.

## Sustained but Not Acute PLC Activation Correlates with $IP_3$ Receptor Ubiquitination

Previous studies have demonstrated that GPCR activation of PLC can induce down-regulation of $IP_3$ receptors ($IP_3Rs$) through the ubiquitin-proteasome pathway (28,36,37), and we have shown that ET-1 stimulates $IP_3R$ ubiquitination and down-regulation in Rat-1 fibroblasts (38). Since sustained PI hydrolysis appears to be required for this process (28,32,37,39), we reasoned that knockdown of PLC isoforms coupled to sustained PI hydrolysis should inhibit $IP_3R$ ubiquitination. Fig. 8 shows the effect of knocking down PLCε or PLCβ3 on type 1 $IP_3R$ ubiquitination stimulated by ET-1 or thrombin. Knockdown of PLCε markedly inhibited ET-1-stimulated ubiquitination and completely blocked the effects of thrombin (Fig. 8, *A* and *B*). In additional experiments, inhibition of ET-1-stimulated ubiquitination was reversed by adenovirus-mediated reconstitution of PLCε, as in Fig. 6 but not with a catalytically inactive mutant, H1433L PLCε, consistent with the lipase function of PLCε mediating agonist-induced $IP_3R$ ubiquitination (Fig. 8*C*). Similarly, knockdown of PLCβ3 partially inhibited ET-1-stimulated ubiquitination, although to a lesser extent than PLCε knockdown and consistent with differences in inhibition of total IP accumulation (see Fig. 3*C*). In contrast, knockdown of PLCβ3 had no effect on thrombin-stimulated ubiquitination (Fig. 8, *A* and *B*), which only inhibited acute PI hydrolysis. These studies show a direct correlation between inhibition of agonist-stimulated sustained PI hydrolysis and $IP_3R$ ubiquitination (see Figs. 3*C* and 8*B*) and that PLCε is predominant in mediating sustained PI hydrolysis.

## DISCUSSION

ET-1, LPA, and thrombin previously have been shown to stimulate cellular PLC activity (25–27). Classically, these and other hormones acting through GPCRs have been proposed to activate PLCβ isoforms through $G_q$ family G-proteins and Gβγ subunits (25–27). In addition, recent studies have raised the possibility that these agonists could activate PLCγ isoforms through transactivation of receptor tyrosine kinase receptors (18,40). The discovery of PLCε, however, raises the possibility that these agonists might activate this novel PLC family member. PLCε has been shown to be regulated by multiple G-proteins, including Ras (4,8,9), Rho (9,10), and $G_{12}$ (5,9) family G proteins and by Gβγ subunits (11). Since ET-1, LPA, and thrombin receptors couple to $G_q$, $G_i$, and $G_{12}$ family G proteins (25–27) and have been shown to activate Ras (18,40–42) and Rho (43), these agonists could potentially activate PLCβ isoforms and/or PLCε, changing the view of how these GPCR agonists regulate cellular PI hydrolysis. To determine whether these agonists couple to PLCβ isoforms and/or PLCε, we identified Rat-1 fibroblasts as a cell line that expresses both of these PLC family members and examined the effects of knocking down specific isoforms on agonist-stimulated PI hydrolysis.

Our studies show that ET-1, LPA, and thrombin, acting through endogenous GPCRs, couple to endogenous PLCε in Rat-1 fibroblasts. This is the first demonstration that these agonists physiologically couple to PLCε. In addition, and consistent with previous paradigms (1,2,25–27), we also show that these diverse agonists activate endogenous PLCβ3. Thus, under physiologic conditions, ET-1, LPA, and thrombin dually regulate both PLCε and PLCβ3 in Rat-1 fibroblasts.

GPCR agonist regulation of these PLC isoforms, however, is not a simple, simultaneous activation of PLCε and PLCβ3. Importantly, we demonstrate that the stimulation of these PLC isoforms is temporally distinct. Our studies show that ET-1, LPA, and thrombin activate PLCβ3 acutely (peak at 15 s; duration ~1 min) but not PLCε. In contrast, ET-1, LPA, and thrombin activate PLCε during sustained PI hydrolysis (>30 s to at least 60 min). This distinct temporal activation of PLCε and PLCβ3 is most apparent for LPA, doses ≤1 μM, and thrombin, which couple to PLCβ3 acutely and almost exclusively to PLCε during sustained stimulation. Consistent with this observation, overexpression of PLCε had no effect on acute but markedly increased sustained agonist-stimulated PI hydrolysis. ET-1 was similarly coupled to PLCβ3 acutely but, in addition to activating PLCε during the sustained phase, also partly activated PLCβ3, indicating an agonist dependence for the temporal activation of these isoforms. Interestingly, a difference in the activation of PLC by these agonists is consistent with an earlier study that noted ET-1 and LPA regulate PLC activity by distinct mechanisms in Rat-1 fibroblasts (44). Overall, however, PLCβ3 predominantly accounts for acute and PLCε for sustained PLC responses. Interestingly, in vascular smooth muscle cells, angiotensin II has been shown to stimulate PLCβ1 acutely followed by activation of PLCγ1 (20), suggesting that acute activation of PLCβ isoforms may be a general phenomenon.

Whereas we have demonstrated that ET-1, LPA, and thrombin regulate PLCβ3 and PLCε, our studies also suggest that these agonists regulate other PLC isoforms. This is apparent, because acute stimulation was only partly inhibited by knockdown of PLCβ3 (Fig. 4) or the combined knockdown of both PLCβ3 and PLCε (Fig. 7). Similarly, high doses (≥3 μM) of LPA activate an unknown PLC during sustained stimulation (Fig. 5*B*). It is possible that either PLCδ1 or PLCγ1, which we show are present in this cell line, mediates these responses. In Rat-1 fibroblasts, ET-1 and LPA have been shown to transactivate the epidermal growth factor receptor (18,40), and therefore PLCγ1 might be activated. In addition, whereas PLCε and PLCβ3 appear to be regulated independently because of their distinct activation profiles and additive effects on cellular PLC activity in combined knockdown studies, other PLC isoforms may be activated downstream of these enzymes. In this regard, PLCβ2 and PLCδ1 have been

suggested to exist as an inactive heterodimer, and upon Gβγ subunit stimulation of PLCβ2, PLCδ1 is released to hydrolyze PIs (21). Whether PLCδ1 is involved in PLCβ3 or PLCε signaling remains to be determined.

Previously, we demonstrated that the GPCR agonists LPA, S1P, and thrombin stimulate PLCε overexpressed in COS-7 cells (9). The present studies confirm that LPA and thrombin couple to PLCε and that the interaction is physiological. Interestingly, in COS-7 cells overexpressing PLCε or PLCβ isoforms, these agonists stimulated PLCε to a markedly greater extent than PLCβ1 or PLCβ2, suggesting preferential coupling to PLCε. However, IP accumulation was measured at 60 min, and acute stimulation was not examined. Because the current studies demonstrate that these agonists regulate PLCβ3 acutely and PLCε in a sustained manner, earlier time points may reveal coupling to PLCβ isoforms.

We have also shown previously that LPA and thrombin activate PLCε through $G\alpha_{12/13}$ and Rap (9). It is possible that a similar mechanism mediates activation of PLCε by thrombin and LPA in Rat-1 fibroblasts, and preliminary studies examining the effects of knocking down $G\alpha_{12/13}$ are consistent with this hypothesis.[3] Thus, differential activation of $G_q$ and $G_{12}$ or Ras family G-proteins may mediate the temporal stimulation of PLCβ3 and PLCε, respectively. Interestingly, we observed that thrombin stimulated PLCε at least 50-fold more potently than PLCβ3 (Fig. 5). Other investigators have found similar differences in the potency for thrombin activation of Rho through $G_{12}$ or $G_{13}$, which is 20-fold greater than activation through $G_q$ (45). Riobo *et al.* (46) have suggested that this difference may be determined by the affinity of the receptor for a given G protein, and this phenomenon may help explain potency differences observed in our studies. Furthermore, our studies also indicate that the signaling pathway(s) underlying thrombin or LPA activation of PLCβ3 desensitize acutely within the first 90 s of stimulation (Fig. 4*B*), as opposed to the pathways regulating PLCε, which remain active for extended periods of at least 60 min (Fig. 3*A*). Whether this difference reflects differential G-protein regulation (*i.e.* $G_q$ *versus* $G_{12/13}$), a functionally different subset of receptors or intrinsic PLC enzymatic regulation remains to be determined.

Consistent with the temporal activation of PLCε and PLCβ3, we demonstrated a direct functional correlation with $IP_3R$ ubiquitination. Certain GPCRs that activate PLC have been shown to induce down-regulation of $IP_3Rs$ (28,36,37), possibly as a physiologic, protective mechanism to prevent overstimulation through the GPCR effector system (36). This down-regulation is mediated by the ubiquitin-proteasome pathway and probably involves an $IP_3$- and $Ca^{2+}$ -induced $IP_3R$ structural change that triggers ubiquitination and subsequent proteasome targeting (36,47). $IP_3$ appears to be required, because microinjection of an $IP_3$ analog causes proteasome-mediated down-regulation (48,49), and mutations in the region of the $IP_3R$ that bind $IP_3$ inhibit ubiquitination (47,50). Indeed, induction of $IP_3R$ ubiquitination and degradation requires sustained activation of PLC (28,32,39). For example, sustained activation of PLC with carbachol in SH-SY5Y neuroblastoma cells (37), cholecystokinin and bombesin in AR4–2J pancreatoma cells (32), or angiotensin II in WB rat liver epithelial cells (39) induces $IP_3R$ ubiquitination and down-regulation. In contrast, agonists that do not activate PLC in a sustained manner, such as carbachol or substance P in AR4–2J cells (32) or epidermal growth factor, vasopressin, or bradykinin in WB cells (39), fail to induce ubiquitination or down-regulation. Our data concur with these studies, since inhibition of sustained, but not acute, PI hydrolysis stimulated by thrombin or ET-1 inhibits $IP_3R$ ubiquitination. Thus, knockdown of PLCε, which has no effect on acute PI hydrolysis but inhibits sustained PI hydrolysis, markedly inhibited thrombin and ET-1 induced ubiquitination, an effect dependent on the lipase function of the PLC (Fig. 8). In contrast, knockdown of PLCβ3 had no effect on thrombin-stimulated ubiquitination and only partly inhibited ET-1, consistent with its effects on PI hydrolysis. This

[3]G. G. Kelley, unpublished observations.

is the first demonstration of the coupling of specific PLC isoforms to this important cellular adaptive response.

The physiologic role of the differential activation of PLCβ3 and PLCε is not known. However, the observed temporal and dose-dependent differences in their activation have multiple implications. Acutely, peak $IP_3$ regulates $Ca^{2+}$ release through the $IP_3R$ (51), and PLCβ3 probably contributes to this response. The potential role for PLCε and sustained PLC activation is less clear. During sustained stimulation, $IP_3$ can be phosphorylated to higher IPs, including inositol 1,3,4,5-tetrakisphosphate and inositol hexakisphosphate, which have been shown to regulate vesicle transport and nuclear transcription factors (52). Similarly, time-dependent production of different DAG species has been noted, which likely have distinct physiologic roles (53). It is intriguing to speculate that sustained agonist activation of PLCε may down-regulate the $IP_3R$ to redirect signaling to these alternative pathways. Furthermore, our studies show a dose-dependent stimulation of PLCε and PLCβ3, which suggests that local agonist concentrations may determine which pathway is activated. This is most apparent for thrombin, which activates PLCε with a potency almost 2 orders of magnitude greater than PLCβ3 (Fig. 5). Thus, concentrations of 3–10 $p_M$ would activate signaling pathways involving PLCε but have little effect on those utilizing PLCβ3. A concentration dependence of ET-1, where concentrations less than 1 $n_M$ promote $Ca^{2+}$ influx but greater stimulate mobilization of intracellular $Ca^{2+}$, has been noted previously in Rat-1 cells (54). Whether differential regulation of PLCε or PLCβ3 accounts for this or other dose-dependent effects remains to be determined. Elucidating the temporally distinct products generated by isoform-specific PLCs and defining their physiologic role is an important area of future investigation.

In summary, our studies demonstrate that the GPCR agonists ET-1, LPA, and thrombin couple to both PLCε and PLCβ3 in Rat-1 fibroblasts. Whereas there is some agonist-dependent overlap, activation of these isoforms occurs in a temporally distinct manner whereby PLCβ3 is activated acutely and PLCε in a sustained manner. This temporal regulation of sustained, but not acute, activation of PLCε and PLCβ3 functionally correlates with $IP_3R$ ubiquitination. In addition, other PLC isoforms, possibly PLCδ1 and/or PLCγ1, are also simultaneously activated. Clearly, these studies demonstrate that GPCR activation of PLC is complex and involves an agonist- and dose-dependent, temporal activation of multiple PLC isoforms, which translates into discrete regulatory functions.

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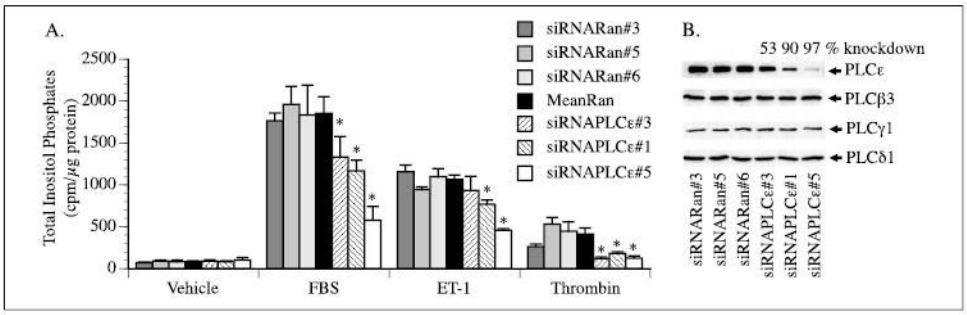


**FIGURE 1. GPCR agonists, endothelin and thrombin, couple to endogenous PLCε through endogenous receptors**

*A*, effect of siRNA-mediated knockdown of PLCε on GPCR stimulation of PLC activity. Rat-1 fibroblasts were stably transduced with retrovirus (pSUPER-Retro) expressing siRNA targeting PLCε (siRNAPLCε#1, #3, or #5) or a random sequence of these siRNAs (siRNARan#3, #5, or #6). Stable cell populations were then stimulated with FBS (10%), ET-1 (100 nM), thrombin (10 nM), or vehicle for 60 min in the presence of 20 mM $Li^+$, and PLC activity was determined. The average of the PLC response in the three random lines is shown (*MeanRan*). Values are mean ± S.E. of three experiments performed in triplicate. *, $p < 0.05$ compared with random controls. *B*, specific knockdown of PLCε. Shown is an image of Western blots of cell lysates stained with anti-PLCε (250 kDa), anti-PLCβ3 (150 kDa), anti-PLCγ1 (145 kDa), or anti-PLCδ1 (85 kDa). Percentage knockdown of PLCε relative to random controls, determined by densitometric measurements of at least three experiments, is shown.

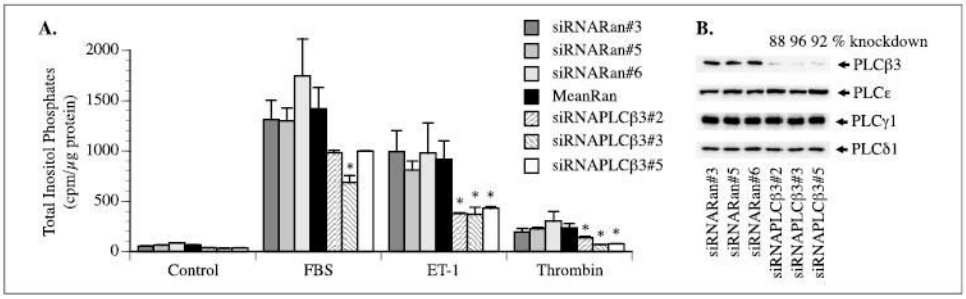


**FIGURE 2. Endothelin and thrombin couple to endogenous PLCβ3**

*A*, effect of siRNA-mediated knockdown of PLCβ3 on GPCR stimulation of PLC activity. Rat-1 fibroblasts were stably transduced with retrovirus (pSUPER-Retro) expressing siRNA targeting PLCβ3 (siRNAPLCβ3#2, #3, or #5) or random sequences (siRNARan#3, #5, or #6). Stable cell populations were then stimulated with FBS (10%), ET-1 (100 nM), thrombin (10 nM), or vehicle for 60 min in the presence of 20 mM $Li^+$, and PLC activity was determined. The average of the PLC response in the three random lines is shown (*MeanRan*). Values are mean ± S.E. of two experiments performed in triplicate. *, $p < 0.05$ compared with random controls. *B*, specific knockdown of PLCβ3. Shown is an image of Western blots of cell lysates stained with anti-PLCβ3 (150 kDa), anti-PLCε (250 kDa), anti-PLCγ1 (145 kDa), or anti-PLCδ1 (85 kDa). Percentage knockdown of PLCε relative to random controls, determined by densitometric measurements of at least three experiments, is shown.

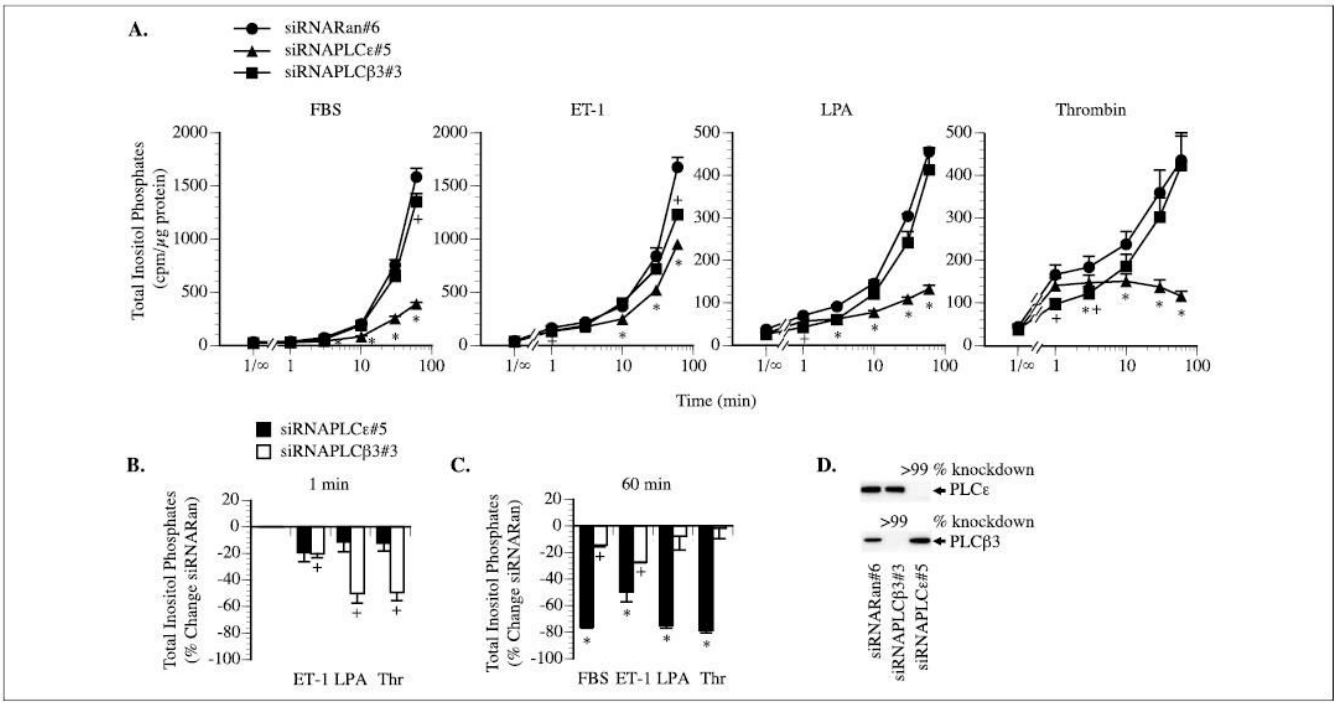


**FIGURE 3. Temporal coupling of GPCR agonist activation of PLCε and PLCβ3**

*A*, effect of siRNA-mediated knockdown of PLCε or PLCβ3 on agonist stimulation of PLC activity. Rat-1 fibroblasts were stably transduced with retrovirus (pSUPER-Retro) expressing siRNA targeting PLCε (siRNAPLCε#5), PLCβ3 (siRNAPLCβ3#3), or a random sequence (siRNARan#6) as in Figs. 1 and 2, except cells were exposed to the retrovirus a second time to increase knockdown. Stable cell populations were then stimulated with FBS (10%), ET-1 (100 nM), LPA (3 μM), thrombin (*Thr*; 10 nM), or vehicle. PLC activity was determined at the indicated times as in Figs. 1 and 2, except the medium was changed to a Krebs buffer, and lithium was increased to 100 mM to prevent metabolism and low IP levels. Values are mean ± S.E. of 3– 6 experiments performed in triplicate. *, $p < 0.05$ for PLCε knockdown cells; +, $p < 0.05$ for PLCβ3 knockdown cells compared with random cells. Shown are relative changes of agonist-stimulated PI hydrolysis induced by knockdown of PLCε or PLCβ3 compared with random treated cells at 1 min (*B*) and 60 min (*C*) time points from *A* (mean ± S.E.). FBS did not stimulate at 1 min. *D*, knockdown of PLCε or PLCβ3. Image of Western blots of cell lysates stained with anti-PLCε (250 kDa) or anti-PLCβ3 (150 kDa). Percentage knockdown of PLCε or PLCβ3 relative to random control, determined by densitometric measurements of at least three experiments, is shown.

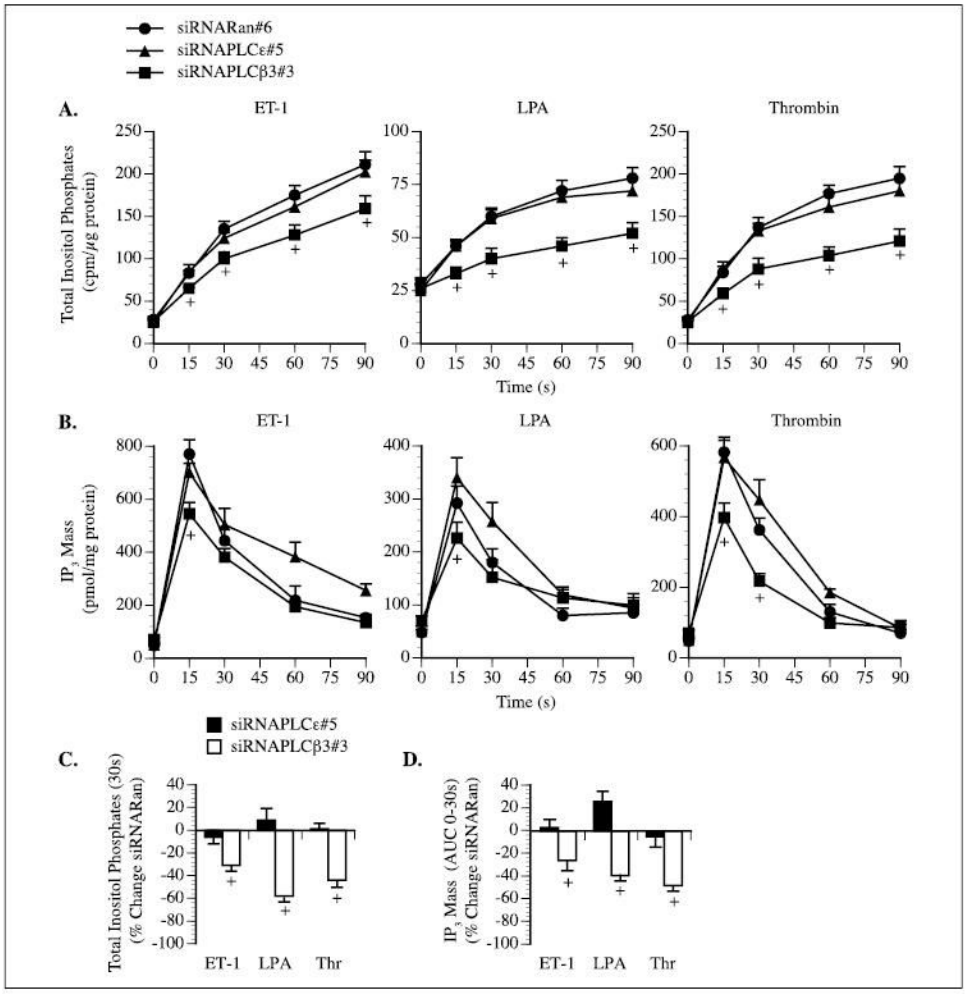


**FIGURE 4. Acute activation of PLCβ3 but not PLCε**

*A*, acute regulation of inositol phosphate production. Control and knockdown Rat-1 fibroblasts described in the legend to Fig. 3 were stimulated with ET-1 (100 nM), LPA (3 μM), thrombin (*Thr*; 10 nM), or vehicle for the indicated times, and acute regulation of total inositol phosphate accumulation in the presence of 100 mM $Li^+$ was determined. Values are mean ± S.E. of four experiments performed in triplicate. *B*, correlation with $IP_3$ mass. In a separate set of experiments, performed similarly but with no added $Li^+$, $IP_3$ mass was measured. Values are mean ± S.E. of four experiments. Shown are relative change of total inositol production measured at 30 s (*C*) and $IP_3$ mass calculated as area under curve (AUC) from 0 to 30 s in PLCε and PLCβ3 knockdown clones compared with random control cells from *A* and *B* (mean ± S.E) (*D*). +, $p < 0.05$ for PLCβ3 knockdown compared with random or PLCε knockdown cell lines.

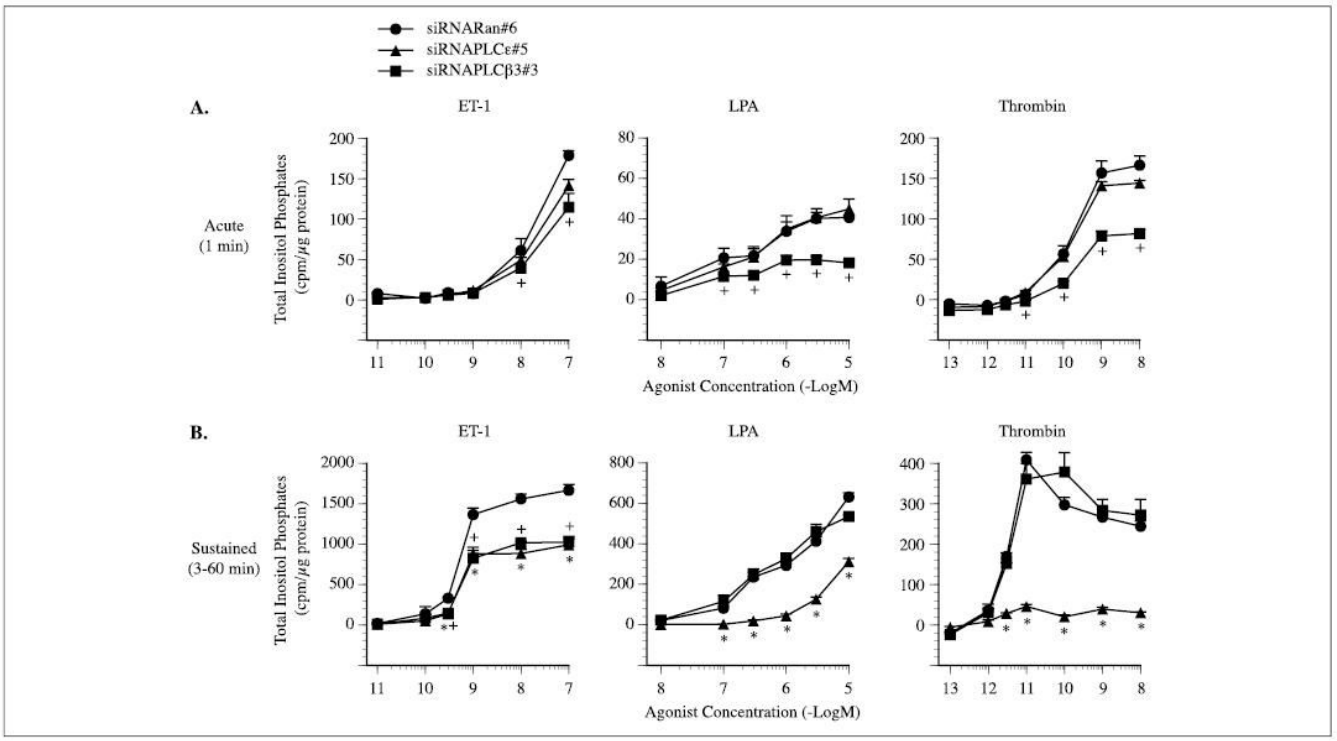


**FIGURE 5. Dose-dependent acute and sustained coupling of GPCR agonists to PLCε and PLCβ3**

Control and knockdown Rat-1 fibroblasts described in the legend to Fig. 3 were stimulated with increasing concentrations of ET-1, LPA, or thrombin. *A*, acute stimulation was determined by preincubating with 100 mM $Li^+$ for 10 min and then adding agonist for 1 min. *B*, sustained stimulation was determined by stimulating with agonist for 60 min as in Fig. 3, except 100 mM $Li^+$ was added 3 min after agonist addition to trap sustained, but not acute IPs. Basal, unstimulated activity (23–25 cpm/μg protein) was subtracted from stimulated responses. Values are mean ± S.E. of 3–5 experiments performed in triplicate. *, $p < 0.05$ for PLCε knockdown; +, $p < 0.05$ for PLCβ3 knockdown compared with random cells.

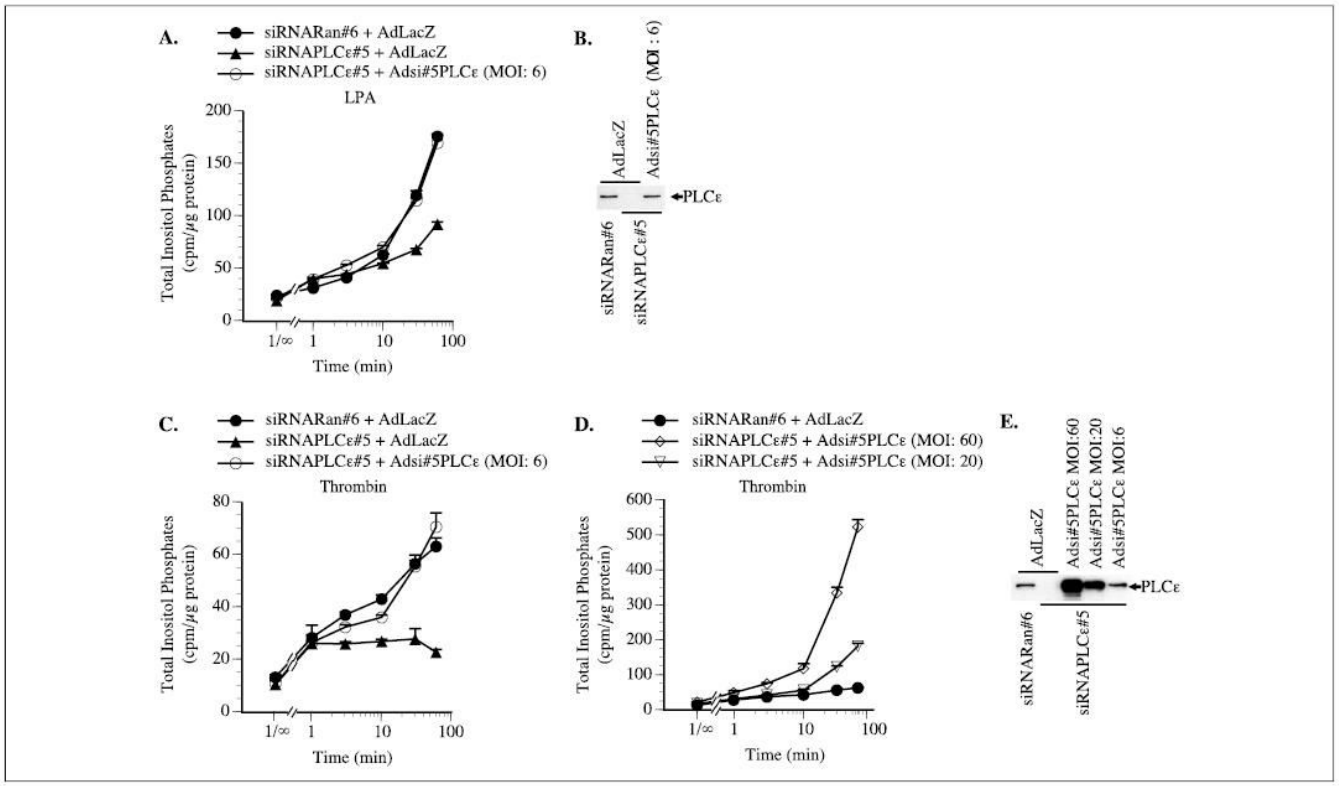


**FIGURE 6. Adenovirus-mediated reconstitution of sustained PLCε activation**

Stable Rat-1 fibroblast cell lines (siRNAPLCε#5 or siRNARan#6 shown in Fig. 3) were transduced with adenovirus expressing LacZ (AdLacZ) or mutant PLCε (Adsi#5PLCε), and PLC activity was determined in response to LPA (3 μM) (*A*) or thrombin (10 nM) (*C* and *D*) as in Fig. 3. The multiplicity of infection (*MOI*; viral plaque-forming units/cell) for ADLacZ was 6.0 and as indicated for PLCε. Values are mean ± S.E. of representative experiments of three similar experiments performed in triplicate for each agonist. Corresponding expression of PLCε in each experiment is shown (*B* and *E*). Western blots of cell lysates were stained with anti-PLCε (250 kDa).

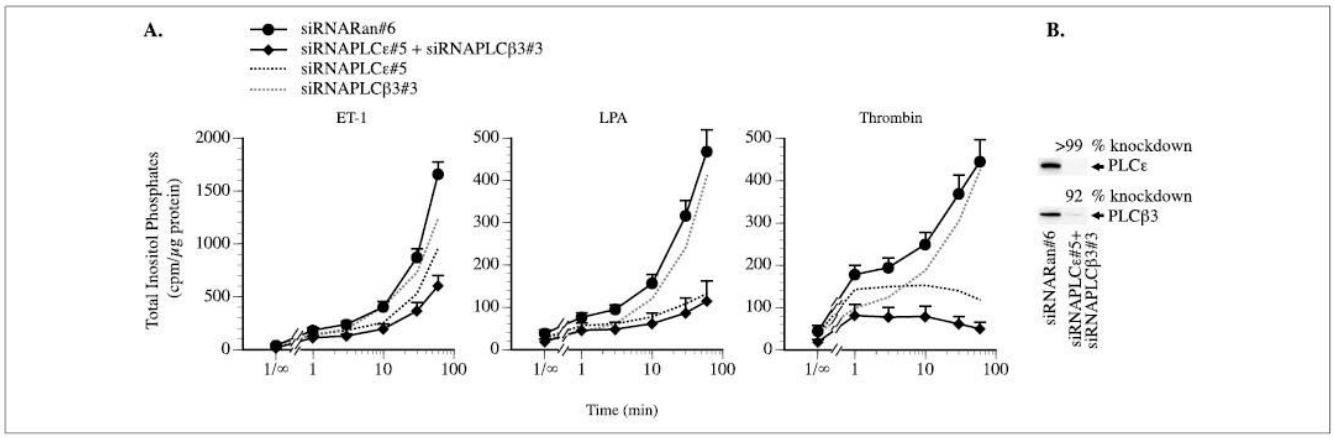


**FIGURE 7. Dual knockdown of PLCε and PLCβ3**

Rat-1 fibroblasts were stably transduced with retrovirus (pSUPER-Retro) expressing siRNA targeting PLCε (siRNAPLCε#5) and PLCβ3 (siRNAPLCβ3#3) or a random sequence (siRNARan#6) (*A*). Stable cell populations were then stimulated with ET-1 (100 nM), lysophosphatidic acid (LPA) (3 μM), thrombin (10 nM), or vehicle, and PLC activity was determined as in Fig. 3. Values are mean ± S.E. of 2–5 experiments performed in triplicate. The *dotted lines* represent values obtained in the single knockdown clones as indicated. *B*, image of Western blots of cell lysates stained with anti-PLCε (250 kDa) or anti-PLCβ3 (150 kDa). Percentage knockdown of PLCε or PLCβ3 relative to random control, determined by densitometric measurements of at least three experiments, is shown.

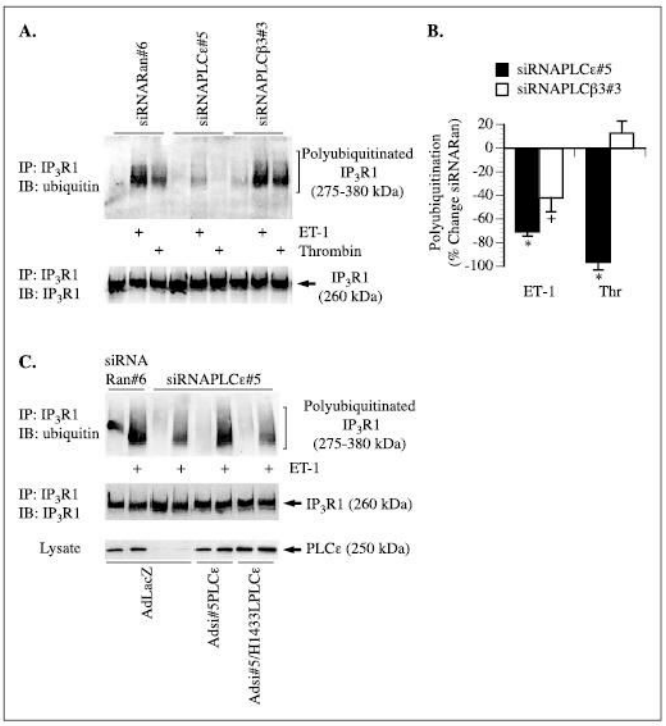


**FIGURE 8. Knockdown of sustained but not acute PI hydrolysis inhibits agonist-mediated $IP_3$ receptor ubiquitination**

*A*, stable PLCε or PLCβ3 knockdown or random siRNA cell lines shown in Fig. 3 were preincubated with 1 μM bortezomib for 1 h and then were exposed to ET-1 (100 nM) or thrombin (*Thr*; 10 nM) for 1 h. $IP_3R1$ was then immuno-precipitated (*IP*), subjected to Western blot analysis (*IB*), and probed with anti-ubiquitin (*upper blot*) and then reprobed with anti-$IP_3R1$ (*lower blot*). Results are representative of four similar experiments. *B*, relative changes of agonist-stimulated ubiquitination of $IP_3R1$ in PLCε or PLCβ3 knockdown cells compared with random cells. Levels of $IP_3R1$ ubiquitination were determined by quantitation of ECL pixel density. Values are mean ± S.E. of 2– 4 experiments. *, $p < 0.05$ for PLCε knockdown cells; +, $p < 0.05$ for PLCβ3 knockdown cells compared with random cells. *C*, reconstitution of ubiquitination. Stable PLCε knockdown or random siRNA cells were transduced with an adenovirus expressing LacZ (AdLacZ), PLCε resistant to siRNAPLCε#5 knockdown (Adsi#5PLCε), or a lipase-inactive mutant, H1433L PLCε (Adsi#5/H1433LPLCε), and $IP_3R1$ ubiquitination in response to ET-1 was determined as in *A*. The *lower blot* shows PLCε expression. Results are representative of two similar experiments.