Activation of Phospholipase C-γ by Phosphatidylinositol 3,4,5-Trisphosphate*

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Signal transduction across cell membranes often involves the activation of both phosphatidylinositol (PI)-specific phospholipase C (PLC) and phosphoinositide 3-kinase (PI 3-kinase). Phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂), a substrate for both enzymes, is converted to phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P₃) by the action of PI 3-kinase. Here, we show that PI(3,4,5)P₃ activates purified PLC-γ isoforms by interacting with their Src homology 2 (SH2) domains. Furthermore, the expression of an activated catalytic subunit of PI 3-kinase in COS-7 cells resulted in an increase in inositol phosphate formation, whereas platelet-derived growth factor-induced PLC activation in NIH 3T3 cells was markedly inhibited by the specific PI 3-kinase inhibitor LY294002. These results suggest that receptors coupled to PI 3-kinase may activate PLC-γ isoforms indirectly, in the absence of PLC-γ tyrosine phosphorylation, through the generation of PI(3,4,5)P₃.

Activation of both PLC and PI 3-kinase often occurs in response to stimulation of cells by a variety of agonists. PLC catalyzes the hydrolysis of PI(4,5)P₂ to generate the second messengers inositol 1,4,5-trisphosphate (I(1,4,5)P₃) and diacylglycerol (1–3). PI 3-kinase phosphorylates the D-3 position of PI(4,5)P₂ to produce PI(3,4,5)P₃, which is then sequentially dephosphorylated to PI(3,4)P₂ and phosphatidylinositol 3-phosphate (4–7). The activation of each of these two enzymes has been implicated in such diverse cellular processes as mitogenesis, chemotaxis, secretion, and cytoskeletal assembly (4–7).

The phosphoinositides PI(3,4)P₂ and PI(3,4,5)P₃ are not substrates of any known PLC (8) and are normally absent from resting cells; however, they appear within seconds to minutes of stimulation of cells with various growth factors or other cellular activators. In contrast, the concentration of phosphatidylinositol 3-phosphate does not change substantially in response to cell stimulation (4–7). It has thus been suggested that PI(3,4,5)P₃ and PI(3,4,5)P₂ might function as intracellular messengers (4–7). With regard to potential targets of these D-3-phosphorylated lipids, they have been shown to activate Ca²⁺-independent isoforms of protein kinase C (9, 10) as well as to bind the pleckstrin homology (PH) domain of the protein serine-threonine kinase Akt, thereby activating its kinase activity (11–13), and to the SH2 domains of the 85-kDa (p85) subunit of PI 3-kinase, thereby preventing its binding to tyrosine-phosphorylated proteins (14).

The 10 mammalian PLC isozymes identified to date are single polypeptides and can be divided into three types: PLC-β, PLC-γ, and PLC-δ (1). All contain a PH domain in their NH₂-terminal region. The γ type isoforms differ from the other two types in that they contain two SH2 domains, one SH3 domain, and an additional PH domain that is split by the SH domains; these domains are arranged in the order PH(N)-SH2-SH2-SH3-PH(C), where N and C in parentheses denote NH₂- and COOH-terminal locations, respectively. Upon stimulation of cells with growth factors like platelet-derived growth factor (PDGF) and epidermal growth factor, the SH2 domain of PLC-γ binds to the autophosphorylated tyrosine residues of growth factor receptors, leading to tyrosine phosphorylation and activation of PLC-γ (1). PLC isoforms can also be activated at least in vitro by the presence of phosphatidic acid (15) or arachidonic acid (16). Therefore, activation of PLC-γ isoforms may occur secondarily to receptor-mediated activation of phospholipase D and cytosolic phospholipase A₂, which results in the production of phosphatidic acid and arachidonic acid, respectively. We now report that another lipid-derived messenger, PI(3,4,5)P₃, activates PLC-γ isoforms specifically by interacting with their SH2 domains.

EXPERIMENTAL PROCEDURES

Materials—PLC isoforms were purified from HeLa cells that had been transfected with recombinant vaccinia virus containing the entire coding sequence of the respective enzyme (17). Dipalmitoyl-PI(3,4,5)P₃ and dipalmitoyl-PI(3,4,5)P₂ were synthesized as described (18). An expression vector (pCMVp110-CAAX) that encodes a fusion protein consisting of Myc epitope and p110-Cα was kindly provided by J. Downward (Imperial Cancer Fund, London, United Kingdom).

PLC Assay—The activities of PLC-β1, PLC-γ1, PLC-γ2, and PLC-δ1 were measured with a mixed micellar substrate containing [⁢⁹⁸⁸⁸]H[/I]PI(3,4,5)P₃, phosphatidylethanolamine, and phosphatidylerine in a molar ratio of 1:3:3 together with various amounts of synthetic dipalmitoyl-PI(3,4,5)P₃ or dipalmitoyl-PI(3,4,5)P₂ in 0.1% deoxycholate. The final assay mixture (100 μl) contained 10 μM [⁹⁸⁸⁸⁸]H[/I]PI(3,4,5)P₃ (26,000 cpm), 50 mM Hepes-NaOH (pH 7.0), 10 mM NaCl, 120 mM KCl, 2 mM EGTA, 0.05% deoxycholate, bovine serum albumin (5 μg/ml), 1 μM free Ca²⁺, and the indicated concentrations of PI(3,4,5)P₃ or PI(3,4,5)P₂. After incu-
bation for 10 min at 30 °C, the reaction was terminated by addition of 200 µl of 10% (w/v) trichloroacetic acid and 100 µl of 10% (w/v) bovine serum albumin, followed by centrifugation. The amount of radioactivity in the resulting supernatant, corresponding to [3H]1,4,5P3, was measured by liquid scintillation spectrometry. The amount of PLC isoforms (4–7 ng) was adjusted to give similar basal activity.

Preparation of GST Fusion Proteins—For each fusion protein (denoted PH(N)-SH2-SH2-SH3-PH(C), SH2-SH2-SH3, SH2-SH2, N-SH2, C-SH2, PH(NC), and SH3), the corresponding polymerase chain reaction product, flanked by BamHI and EcoRI linkers, was inserted into the BamHI and EcoRI sites of the glutathione S-transferase (GST) expression vector pGEX-2TK (Pharmacia Biotech Inc.). Amino acid sequences of PH(N)-SH2-SH2-SH3-PH(C), SH2-SH2-SH3, SH2-SH2, N-SH2, C-SH2, and SH3 correspond to residues 483–936, 533–851, 550–745, 550–657, 668–745, and 758–851 of PLC-γ1, respectively. Polymerase chain reaction products corresponding to residues 482–523 (PH(N)) and 865–936 (PH(C)) of PLC-γ1 were fused with a glycine codon insertion to yield the combined PH(NC) construct. The 5’ and 3’ primers for PH(N) contained BamHI and Smal sites, respectively; thus, the PH(NC) construct contained sequential BamHI, Smal, and EcoRI sites. Escherichia coli cells were transformed with the various expression vectors and cultured at 30 °C. Expression of the GST fusion proteins was induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside, and the cells were subsequently collected by centrifugation at 2000 × g for 15 min, sonicated in phosphate-buffered saline, and centrifuged at 5000 × g for 15 min. The resulting supernatant was mixed and incubated at room temperature for 30 min with 2 ml of a 50% (v/v) slurry of glutathione-Sepharose 4B (Pharmacia) that had been equilibrated with phosphate-buffered saline. After centrifugation of the mixture at 5000 × g for 15 min, the supernatant was removed and the pellet washed with 10 bed volumes of phosphate-buffered saline. Bound proteins were cleaved from GST by incubation of the beads with thrombin (10 µg/ml) at room temperature for 6 h. The eluted proteins were further purified by high performance liquid chromatography on a Mono Q column and quantitated spectrophotometrically with extinction coefficients at 280 nm calculated on the basis of their amino acid composition.

RESULTS AND DISCUSSION

We investigated the effects of PI(3,4)P2 and PI(3,4,5)P3 on the activities of PLC isozymes by measuring the hydrolysis of PI(4,5)P2 with mixed micellar substrates containing phosphatidylethanolamine, phosphatidylserine, [3H]PI(4,5)P2, and various amounts of synthetic D-3-phosphorylated lipids. PI(3,4,5)P3 increased the activities of PLC-γ1 and PLC-γ2 but had no effect on PLC-β1 or PLC-β1 (Fig. 1A). The dependence of the activities of PLC-γ1 and PLC-γ2 on PI(3,4,5)P3 concentration was sigmoidal, with maximal activation (approximately 8-fold) apparent at 100 µM lipid. In contrast, PI(3,4)P2 had no effect on the activities of any of the PLC isoforms examined (Fig. 1B).

We prepared various recombinant proteins containing different domains of PLC-γ1 (Fig. 2, A and B) and measured their effects on the activity of full-length PLC-γ1 in the presence of 100 µM PI(3,4,5)P3. All SH2 domain-containing proteins inhibited the PI(3,4,5)P2-stimulated activity of PLC-γ1 in a concentration-dependent manner, whereas proteins corresponding to the SH3 and PH(NC) (fusion of PH(N) and PH(C)) domains had no effect (Fig. 2C). The inhibition by SH2 proteins was apparent only in the presence of PI(3,4,5)P3, the basal activity of PLC-γ1 being unaffected (Fig. 2D). These results suggest that PI(3,4)P2 activates PLC-γ isoforms by binding to their SH2 domains. Furthermore, the three proteins PH(N)-SH2-SH2-SH3-PH(C), SH2-SH2-SH3, and SH2-SH2, all of which contain two SH2 domains, inhibited PI(3,4,5)P3-dependent PLC-γ1 activity to a greater extent than did the NH2-terminal SH2 (N-SH2) or COOH-terminal SH2 (C-SH2) domains alone. This result, together with the sigmoidal response of PLC-γ isozyme activities to PI(3,4,5)P3 indicates that the two SH2 domains bind PI(3,4,5)P3 with positive cooperativity or that the two PI(3,4,5)P3-bound domains mediate enzyme activation synergistically. The ability of PI(3,4,5)P2 but not PI(3,4)P2 to activate PLC-γ1 via its SH2 domains is consistent with the previous observation that p85, Src, and Abl SH2 domains show higher affinity for PI(3,4)P2 than for PI(3,4,5)P3 or PI(4,5)P2 (14). PLC-γ isoforms contain an additional PH domain near their NH2 terminus. Recent results by Falasca et al. (19) suggest that PI(3,4,5)P3 may also bind to the PH domain (19).

The concentration of PI(3,4,5)P3 required for activation of PLC-γ isoforms in vitro is relatively high. However, it has been suggested that the intracellular concentration of PI(3,4,5)P3 can achieve values of up to 200 µM in neutrophils stimulated with formylmethionyl-leucyl-phenylalanine (fMLP) (20). Specific generation of PI(3,4,5)P3 at sites of PLC-γ localization might be one means of ensuring effective activation of PLC-γ isoforms. The sigmoidal response to PI(3,4,5)P3 also suggests that PLC-γ activation would be minimal until the lipid concentration exceeds a certain threshold.

To determine whether activation of PLC by PI(3,4,5)P3 could be detected in intact cells, we transiently expressed in COS-7 cells the 110-kDa subunit (p110) of PI 3-kinase with c-Myc epitope and farnesylation signal (CAAX) sequences located at the NH2 and COOH termini, respectively (21), and measured the release of inositol phosphates resulting from the hydrolysis of PI(4,5)P2. Expression of the Myc-tagged p110-CAAX protein was detected by immunoblot analysis with antibodies specific to the Myc sequence (Fig. 3A). The farnesylation signal se-
PLC-γ and PI 3-Kinase

Fig. 2. Effects of various recombinant PLC-γ1 domain-containing proteins on PI(3,4,5)P3-dependent PLC-γ1 activity. A, schematic representation of the recombinant PLC-γ1 constructs. The top scheme shows the SH2, SH3, and split PH [PH(N) and PH(C)] domains of PLC-γ1 flanked by the X and Y catalytic domains. The recombinant constructs shown below were expressed as GST fusion proteins in E. coli, purified, and cleaved from GST. The PH(NC) construct comprises the fused PH(N) and PH(C) domains with an inserted glycine residue between them. B, analysis of the purity of the recombinant PLC-γ1 protein preparations. The purified proteins were analyzed by SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining. Lane 1, PH(N)-SH2-SH2-SH3-PH(C); lane 2, SH2-SH2-SH3; lane 3, SH2-SH2, lane 4, NH2-terminal SH2 (N-SH2); lane 5, COOH-terminal SH2 (C-SH2); lane 6, PH(NC); lane 7, SH3. The positions of molecular size standards (in kilodaltons) are shown on the left. C, effects of recombinant proteins on the activity of full-length PLC-γ1 in the presence of PI(3,4,5)P3. The hydrolysis of [3H]PI(4,5)P2 by 1.1 nM PLC-γ1 was measured in the presence of the indicated concentrations of the various recombinant proteins and 100 μM PI(3,4,5)P3, as described under "Experimental Procedures." D, effects of recombinant proteins on basal PLC-γ1 activity. The activity of PLC-γ1 was assayed as in C but in the absence of PI(3,4,5)P3. Data in C and D are means of duplicate measurements from a single experiment and are representative of three similar experiments.


cquence causes the constitutive activation of p110 by targeting it to the cell membrane. As expected from the fact that most mammalian cells contain a relatively high concentration (>20 μM) of inositol phosphates even before stimulation (22), a substantial amount of [3H]labeled inositol phosphates was detected in COS-7 cells labeled with [3H]inositol to equilibrium (Fig. 3B). Expression of Myc-p110-CAAX induced a 45% increase in the amount of inositol phosphates, and this effect was blocked by pretreatment of cells with LY294002, a specific inhibitor of PI 3-kinase (23). LY294002 had no effect on the amount of inositol phosphates in cells not expressing Myc-p110-CAAX. These results suggest that PI(3,4,5)P3, generated by the activated p110 subunit, was able to activate PLC. The increase in PLC activity was smaller than that in cells stimulated with PLC-activating agonists, probably because in the local area where PLC-γ was activated by PI(3,4,5)P3, PI(4,5)P2 (a common substrate for PLC and PI 3-kinase) had been depleted by Myc-p110-CAAX. It has been shown previously that availability of PI(4,5)P2 is a limiting factor for PLC activity (24).

Further evidence that PI(3,4,5)P3 activates PI(4,5)P2 hydrolysis in intact cells is provided by previous studies with wortmannin, a potent (median inhibitory concentration (IC50), 3 nM), irreversible inhibitor of PI 3-kinase (23). Wortmannin inhibits I(1,4,5)P3 formation and Ca2+ mobilization in bovine adrenal glomerulosa cells stimulated by angiotensin II (25), rat basophilic leukemia (RBL-2H3) cells stimulated by cross-linking of high affinity immunoglobulin E (IgE) receptors (26), and human neutrophils stimulated by fMLP (27). At the time of the studies with neutrophils and adrenal glomerulosa cells, wortmannin was known to inhibit myosin light chain kinase (MLCK), but its effect on PI 3-kinase was not known. The inhibition of I(1,4,5)P3 and Ca2+ responses by 20 nM wortmannin in adrenal glomerulosa cells was thus speculated to result from inhibition of MLCK. However, it is now known that the concentration of wortmannin required for inhibition of MLCK is 100 times that required for inhibition of PI 3-kinase (23), so that MLCK was likely not inhibited by 20 nM wortmannin. Our data indicate that they are likely attributable to prevention of PI(3,4,5)P3-induced activation of PLC-γ. PLC-γ1 is a widely expressed and abundant enzyme, whereas PLC-γ2 is abundant in cells of hematopoietic origin.

Recently, type III PI 4-kinase was shown to be inhibited by wortmannin (IC50, 50 nM) (23). Because PI 4-kinase is required for PI(4,5)P2 synthesis, it is possible that the reduced activity of PLC observed in wortmannin-treated cells was attributable to the diminished supply of substrate. Thus, we studied the effect of the more specific inhibitor LY294002 (IC50, 2 and 100 μM for PI 3-kinase and type III PI 4-kinase, respectively; Ref. 23) on PI(4,5)P2 hydrolysis induced by PDGF in NIH 3T3 cells. Whereas PDGF induced an 8-fold increase in PLC activity in
isoforms by G proteins (1–3). Tyrosine phosphorylation of PLC-γ requires SH2 domain-mediated association with a tyrosine-phosphorylated PTK. Because PI(3,4,5)P3 competes with tyrosine-phosphorylated proteins for binding to SH2 domains, an increase in PI(3,4,5)P3 concentration might attenuate PTK-dependent activation of PLC-γ.

PI 3-kinase exists in two types: one that is activated by PTKs and one activated by G proteins (1–3). Although only 30 receptors are currently known to stimulate PLC-γ, most of these also activate PLC. There-
PTK-coupled receptors such as the IgE receptor. The fact that wortmannin inhibits PLC activation induced by G protein-coupled receptors such as those for angiotensin II and MLP suggests that PLC-γ might be activated indirectly in response to the occupancy of such receptors. However, the activation of PI 3-kinase appears not always to result in activation of PLC-γ, because there is no evidence that insulin or colony-stimulating factor-1, both of which activate PI 3-kinase, elicits the production of I(1,4,5)P₃ (31, 32). One possible explanation is that PLC-γ isozymes may not be located at the sites where PI(3,4,5)P₃ is generated in the cells activated with colony-stimulating factor or insulin.

Finally, our results may explain how engagement of the FcεRIIB inhibitory receptor in mast cells reduces the IgE-induced increase in intracellular Ca²⁺ concentration (33). FcεRIIB binds SHIP, a phosphatase that dephosphorylates the D-5 position of PI(3,4,5)P₃ or I(1,3,4,5)P₄. A decrease in the concentration of PI(3,4,5)P₃ could reduce I(1,4,5)P₃ production by PLC-γ isozymes and thereby reduce long term release of intracellular Ca²⁺ and ultimately Ca²⁺ influx.

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REFERENCES

1. Rhee, S. G., and Bae, Y. S. (1997) J. Biol. Chem. 272, 10245–10248
2. Cockcroft, S., and Thomas, G. M. H. (1992) Biochem. J. 286, 1–14
3. Berridge, M. J. (1993) Nature 361, 315–325
4. Cantley, L., Auger, K. R., Carpenter, C., Duckworth, B., Graziani, R., Kapeller, R., and Soltoff, S. (1991) Cell 64, 281–302
5. Fry, M., and Waterfield, M. D. (1993) Phil. Trans. R. Soc. London Series B 340, 337–344
6. Stephens, L., Jackson, T., and Hawkins, P. (1993) Biochem. Biophys. Acta 1179, 27–75
7. Kapeller, R., and Cantley, L. (1994) BioEssays 16, 565–576
8. Serunian, L. A., Haber, M. T., Fukui, T., Kim, J. W., Rhee, S. G., Lowenstein, J. M., and Cantley, L. C. (1988) J. Biol. Chem. 263, 17899–17905
9. Nakanishi, H., Brewer, K. A., and Exton, J. H. (1993) J. Biol. Chem. 268, 13–16
10. Toker, A., Meyer, M., Reddy, K. K., Falch, J. R., Aneja, R., Aneja, S., Parra, A., Burns, D. J., Ballas, L. M., and Cantley, L. C. (1994) J. Biol. Chem. 269, 32358–32367
11. Franke, T. F., Yang, S. I., Chan, T. O., Datta, K., Kazlauskas, A., Morrison, D. K., Kaplan, D. R., and Tsichlis, P. N. (1995) Cell 81, 727–736
12. Franke, T. F., Kaplan, D. R., Cantley, L. C., and Toker, A. (1997) Science 275, 665–668
13. Stokoe, D., Stephens, L. R., Copeland, T., Gaffney, P. R., Reese, C. B., Painter, G. F., Holmes, A. B., McCormick, F., and Hawkins, P. T. (1997) Science 277, 567–570
14. Rameh, L., Chen, C.-S., and Cantley, L. C. (1995) Cell 83, 821–830
15. Jones, G. A., and Carpenter, G. (1993) J. Biol. Chem. 268, 20845–20850
16. Hwang, S. C., Jhun, D.-Y., Bae, Y. S., Kim, J. H., and Rhee, S. G. (1996) J. Biol. Chem. 271, 18342–18349
17. Park, D., Jhun, D.-Y., Kriz, R., Knopf, J., and Rhee, S. G. (1992) J. Biol. Chem. 267, 16048–16055
18. Wang, D.-S., and Chen, C.-S. (1996) J. Org. Chem. 61, 5905–5906
19. Falasca, M., Logan, S. K., Lehtio, V. P., Baceante, G., Lemmon, M. A., and Schlessinger, J. (1998) EMBO J., in press
20. Stephens, L. R., Hughes, K. T., and Irvine, R. F. (1991) Nature 351, 33–39
21. Khwaja, A., Rodriguez-Viciana, P., Wrennstrom, S., Warne, P. H., and Downward, J. (1997) EMBO J. 16, 2783–2793
22. Stokoe, D., Graham, R. A., and Brown, T. R. (1987) Biochem. Biophys. Res. Commun. 149, 874–881
23. Downing, G. J., Kim, S., Nakanishi, S., Catt, K. J., and Balla, T. (1996) Biochemistry 35, 5857
24. Cunningham, E., Tan, S. K., Swigart, P., Hsuau, J., Bankaitis, V., and Cockcroft, S. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 6589
25. Nakanishi, S., Catt, K. J., and Balla, T. (1994) J. Biol. Chem. 269, 6528–6535
26. Baker, S., Caldwell, K. K., Hall, A., Martinez, M., Pfeiffer, J. R., Oliver, J. M., and Wilson, B. S. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 939–943
27. Bonser, R. W., Thompson, N. T., Randall, B. W., Tateson, J. E., Spacey, G. D., Hodson, H. F., and Garland, L. G. (1991) Br. J. Pharmacol. 103, 1237–1241
28. Stoyanova, S., Vanhaesebroeck, B., and Dhand, R. (1995) EMBO J. 24, 32358–32367
29. Rameh, L., Chen, C.-S., and Cantley, L. C. (1995) Cell 83, 821–830
30. Thomason, P., James, S. R., Casey, P. J., and Downes, C. P. (1994) J. Biol. Chem. 269, 16525–16528
31. Whetton, A. D., Monk, P. N., Consalvey, S. D., and Downes, C. P. (1986) EMBO J. 5, 3281–3286
32. Nishibe, S., Wahl, M. I., Wedegaertner, P. B., Kim, J. W., Rhee, S. G., and Carpenter, G. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 424–428
33. Ono, M., Bolland, S., Tempst, P., and Ravetch, J. V. (1996) Nature 383, 262–269
34. Downes, C. P., Hawkins, P. T., and Irvine, R. F. (1986) Biochem. J. 238, 501–506
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