Vol. 163, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1989 Pages 177-182

CYCLIC AND NONCYCLIC INOSITOL PHOSPHATES ARE FORMED AT DIFFERENT RATIOS BY PHOSPHOLIPASE C ISOZYMES

Jae Won Kim, Sung Ho Ryu, and Sue Goo Rhee

Laboratory of Biochemistry
National Heart, Lung, and Blood Institutes
National Institutes of Health
Bethesda, Maryland 20892

Received June 28, 1989

SUMMARY: The cyclic inositol phosphate content in the product of PLC-ß, γ, and δ mediated cleavage of three phosphoinositides, PtdIns, PtdIns(4)p, and PtdIns(4,5)P_2, was measured under several different experimental conditions. The ratio of cyclic to noncyclic product generally decreased in the order PLC-ß > PLC-δ > PLC-γ. For all three enzymes the ratio decreased in the order PtdIns > PtdIns(4)P > PtdIns(4,5)P_2. For all combinations of the three enzymes and three substrates cyclic product content was always higher at pH 5.5 than at pH 7.0. The effect of Ca^{2+} on the ratio of cyclic to noncyclic was also measured. The ratio remained constant between 0.5 μM and 2 mM for PtdIns. For PtdIns(4)P and PtdIns(4,5)P_2, the ratios were unchanged between 0.5 and 500 μM, but increased abruptly at millimolar Ca^{2+} concentrations. © 1989 Academic Press, Inc.

Occupancy of a variety of cell surface receptors by the appropriate ligands results in the phospholipase C-mediated cleavage of PtdIns(4,5)P_2 and generates intracellular messenger molecules such as Ins(1,4,5)P_3 and diacylglycerol (1-3). Ins(1,4,5)P_3 binds to a specific receptors to release stored Ca^{2+} (1-4) whereas diacylglycerol activates protein kinase C to increase protein phosphorylation (1,2). All PtdIns-specific phospholipase Cs (PLC) purified up until now hydrolyze the three inositol-containing phospholipids, PtdIns, PtdIns(4)P, and PtdIns(4,5)P_2 to produce Ins(1)P, Ins(1,4)P_2, and Ins(1,4,5)P_3, respectively, with diacylglycerol as a common product (5-11). Also produced are cyclic inositol phosphates containing a phosphodiester bond between the hydroxyl groups at 1 and 2 positions (12-14). When the 2-hydroxyl group of inositol participates in the cleavage of the diester bond between inositol phosphate and diacylglycerol moieties, 1:2-cyclic inositol phosphates are formed. On the other hand, participation of a free hydroxide ion in the cleavage reaction leads to the noncyclic products.

Abbreviations Used: PLC, phosphoinositide-specific phospholipase C; PtdIns, phosphatidylinositol; PtdIns(4)P, phosphatidylinositol 4-phosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; Ins(1)P, Ins(1,4)P_2, Ins(1,4,5)P_3, and Ins(1,3,4,5)P_4, inositol mono-, bis-, tris-, and tetrakis-phosphate, with the location of phosphate group in parenthesis; cycIns(1-2,4,5)P_3, inositol 1,2-cyclic 4,5-trisphosphate.

0006-291X/89 $1.50
Copyright © 1989 by Academic Press, Inc.
All rights of reproduction in any form reserved.

There is now considerable evidence that cycIns(1–2,4,5)P_3, as well as Ins(1,4,5)P_3, is formed upon activation of a variety of cells and tissues by the proper calcium–mobilizing agonist (15–21). However, the kinetics of cycIns(1–2,4,5)P_3 accumulation is significantly different from that of Ins(1,4,5)P_3 (17–22). Like most other second messengers, Ins(1,4,5)P_3 is generated rapidly in response to an agonists and metabolized rapidly; it is either dephosphorylated to Ins(1,4)P_2 by Ins(1,4,5)P_3–5–phosphatase (23,24) or phosphorylated to Ins(1,3,4,5)P_4 by Ins(1,4,5)P_3–3–kinase (23–25). However, cycIns(1–2,4,5)P_3 is a poor substrate for both the 5–phosphatase and 3–kinase, and degrades very slowly (28,29). Despite this slow turnover cycIns(1–2,4,5)P_3 had been considered to be another calcium–mobilizing second messenger in its own right, because cycIns(1–2,4,5)P_3 was equipotent to Ins(1,4,5)P_3 in releasing Ca^{2+} from permeabilized platelets (30) and from Swiss 3T3 cells (31). However, two recent experiments by Willcocks et al. (32) and by Lee and Hokin (33) suggest that the potency of cycIns(1–2,4,5)P_3 in mobilizing Ca^{2+} is about an order of magnitude less than that of Ins(1,4,5)P_3. In addition, Hughes et al. (20) concluded, after a careful analysis of the kinetics of cycIns(1–2,4,5)P_3 metabolism in rat parotid acinar cells, that the rates of formation and turnover of cycIns(1–2,4,5)P_3 are too slow to be the primary signal for the regulation of intracellular Ca^{2+}. The physiological role of cyclic inositol phosphate is not known. Nevertheless, based on the fact that cycIns(1–2,4,5)P_3 accumulates to a significant concentration (often higher than that of Ins(1,4,5)P_3) at the late stage of cell activation, its regulatory role was proposed to be related to an unknown cellular response (20,33).

Recently, three immunologically distinct PLC enzymes (PLC–ß, γ, and δ; see reference 34 for nomenclature) have been purified (6). The three isozymes are quite dissimilar in molecular size (ß, 150000; γ, 145000; δ, 85000) and in amino acid sequences (39), and expressed differently between tissues and in individual cells (36,37). Since cycIns(1–2,4,5)P_3 declines very slowly, the accumulation rate would mainly depend on the rate of formation. With the aim of investigating the possibility that activation of different PLC isozymes might cause the production of cyclic and noncyclic inositol phosphates in different proportions, the inositol phosphates generated from PtdIns, PtdIns(4)P and PtdIns(4,5)P_2 by three purified PLC isozymes have been analyzed here.

MATERIALS AND METHODS

Materials. Bovine brain PLC–ß, PLC–γ, and PLC–δ were purified as described (36). [^{3}H]PtdIns, [^{3}H]PtdIns(4)P, [^{3}H]PtdIns(4,5)P_2, [^{3}H]Ins(1)P, [^{3}H]Ins(1,4)P_2, and [^{3}H]Ins(1,4,5)P_3 were obtained from New England Nuclear. Soybean PtdIns, brain PtdIns(4)P, and brain PtdIns(4,5)P_2 were purchased from Sigma Chemicals. [^{3}H]cycIns(1–2,4,5)P_3 was kindly provided by Dr. L.E. Hokin.

Phospholipase C Reaction. The reaction mixture included a mixture of 40,000 cpm of [^{3}H]phosphoinositide and 150 μM unlabeled phosphoinositide, increasing amounts of $CaCl_2$ in 200 ml of buffer containing 50 μM Tris–maleate (pH 5.5 or 7.0), 0.1% sodium

deoxycholate, and 2 μM EGTA. Free Ca^{2+} concentrations were calculated as described (36). The reaction was started by adding PLC and incubated for 15 min at 37°C. The amounts of enzyme were adjusted to attain 5–20% of phosphoinositide hydrolysis.

Separation of Inositol Phosphates. The PLC reaction was stopped by adding 1 ml of methanol/chloroform (1:1). Phase separation was achieved by adding 0.25 ml of water and the upper layer was lyophilized. Dried material was redissolved in 0.5 ml of water and passed through Sep-Pak C18 cartridge (Waters Associates, Milford, MA) to remove residual phosphoinositide. The cartridge was eluted with 2 ml of water. The entire sample was applied to a Partisil 10 SAX Column C (Whatman) and eluted with ammonium phosphate (pH 3.8) gradient as described (38). The ^3H-radioactivity was detected and integrated by using an on-line radio-flow detector (Radiomatic Instruments, Tampa, FL) equipped with an integrator. Excellent separation of cyclic and noncyclic inositol phosphates was achieved. Cyclic inositol phosphate peak eluted before the peak of corresponding noncyclic form. The position of noncyclic inositol phosphates and cycIns(1–2,4,5)P_3 were validated by comparing to those of authentic sample. In addition, to confirm that the putative cyclic inositol phosphate peak was in fact cyclic phosphodiester, the sample was hydrolyzed with 0.2 M HCl at 80°C for 3 min prior to HPLC analysis. This acid treatment abolished the putative cyclic peak and increased the noncyclic peak.

RESULTS

Cyclic inositol phosphate measured at various Ca^{2+} concentrations is presented as a percentage of total inositol phosphate (cyclic + noncyclic) in Figure 1. The percentage of cyclic inositol phosphate decreased in the order PLC-ß > PLC-δ > PLC-γ (except the PtdIns cleavage for which PLC-δ generates slightly more cycIns(1)P than PLC-ß); for example, the average value of cycIns(1–2,4,5)P_3 produced below 500 μM Ca^{2+} by PLC-ß, PLC-δ, and PLC-γ was 10.3, 8.7, and 1.9%, respectively, at pH 5.5. and 5.3, 3.7, and 0.6%, respectively, at pH 7.0. The percentage of cyclic product also decreased in the order PtdIns > PtdIns(4)P > PtdIns(4,5)P_2 for the three enzymes; for example, in the PLC-ß dependent reaction at pH 7.0 and below 500 μM Ca^{2+}, the average percentage of cyclic form decreased from 70.0% for PtdIns to 12.3% for PtdIns(4)P, to 5.3% for PtdIns(4,5)P_2. Cyclic form was always higher at pH 5.5. than at 7.0. The cycIns(1)P formation form PtdIns was independent of Ca^{2+} between 0.5 μM and 2 μM for the three PLC isozymes. The cyclic percentage was also constant between 0.5 and 500 μM of Ca^{2+} when PtdIns(4)P and PtdIns(4,5)P_2 were sued as substrates but increased rapidly for all three enzymes when the Ca^{2+} concentration was increased to 2 μM.

DISCUSSION

Previously, we have shown that three PLC isozymes—PLC-ß, γ, and δ—are differently distributed between and within tissues PLC-ß is highly concentrated in the brain but nearly absent in the liver and lung (37). In addition, within the brain PLC-ß and PLC-δ are localized in specific neurons and in astroglial cells, respectively, while PLC-γ is rather ubiquitous (36). Our present study demonstrates that the concentration of

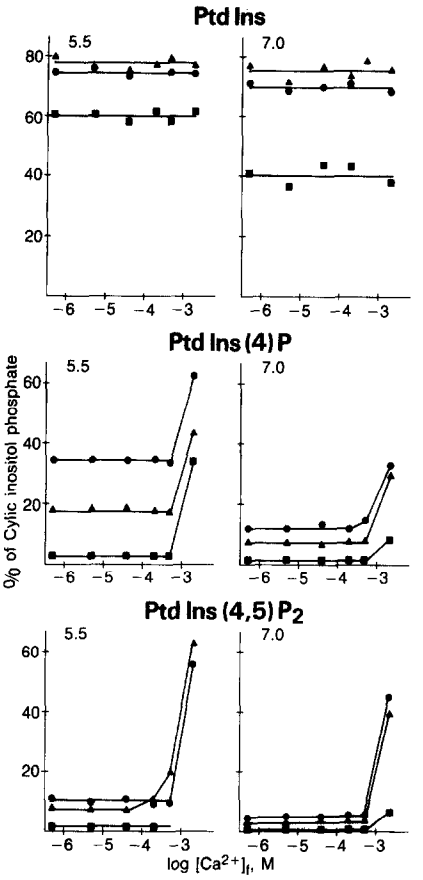

Figure 1. Percentage of cyclic inositol phosphate formed at various Ca^{2+} concentrations. PLC-ß (●---●), PLC-γ (■---■), and PLC-δ (▲---▲) were used to cleave PtdIns, PtdIns(4)P, and PtdIns(4,5)P_2 at pH 5.5 (left column) and pH 7.0 (right column).

cyclic inositol phosphate relative to the noncyclic form can be widely varied depending on which form of PLC isozyme is activated. At pH 7.0, the percentage of cycIns(1-2,4,5)P_3 produced by PLC-γ is 1/9 and 1/6 of that produced by PlC-ß and PLC-γ, respectively. Indeed, the percentage of cycIns(1-2,4,5)P_3 measured relative to Ins(1,4,5)P_3 after treatment with the proper agonist varied widely for different tissues; it was 17% (10 sec) and 36% (30 sec) in platelets (21), 5% (10 sec) and 33% (5 min) in rat parotid gland (19), and 5% (3 min) in WRK-1 cells (22).

Wilson et al. (14) have shown that cyclic inositol phosphate can be generated by the action of two PLC enzymes (65 and 85 KDa) obtained from sheep seminal vesicular gland. In their study, the cyclic form was quantitated by mass spectral analysis of ^{18}O content after the hydrolysis of cyclic form in $H_2{}^{18}O$. For both PLC enzymes, the ratio of cyclic to noncyclic product decreased in order PtdIns > PtdIns(4)P > PtdIns(4,5)P_2. Despite the wide range of experimental error, the mass spectral data also showed that 65 KDa enzyme

produces more cyclic forms than 85 KDa PLC on the average. However, Wilson et al. did not draw attention to this probably because the reproducibility of mass spectral analysis is not good enough to warrant this conclusion. Recent immunological studies show that the 65 KDa enzyme (PLC-α) is distinct from the three PLC enzymes (ß, γ, and δ), while the 85 KDa enzyme is probably the same as PLC-δ (34).

As noted by Dawson et al. (12), the 2-OH group of inositol or a free OH^- attacks the phosphorus atom between inositol and diacylglycerol moieties leading to the cyclic and noncyclic product, respectively. Increased formation of the cyclic form at acidic pH is due to the decrease in free OH^-. Sequential introduction of negative charge into the inositol ring by phosphorylating the 4-OH and 5-OH gradually reduces the participation of the 2-OH in the cleavage reaction. Current results show that the binding of Ca^{2+} to the phosphate groups recuperates the 2-OH group participation in the cleavage reaction. The millimolar concentration of Ca^{2+} required to enhance the formation of cyclic product probably reflects the binding affinity of Ca^{2+} to the phosphate in the inositol ring.

REFERENCES

1. Berridge, M.J. (1987) Annu. Rev. Biochem. 56, 159-193.
2. Hokin, L.E. (1985) Annu. Rev. Biochem. 54, 205-235.
3. Majerus, P.W., Connolly, T.M., Deckmyn, H., Ross, T.S., Bross, T.E., Ishii, H., Bansal, V.S., and Wilson, D.B. (1986) Sicnece 234, 1519-1526.
4. Irvine, R.F., Moor, R.M., Pollock, W.K., Smith, P.M., and Wreggett, K.A. (1988) Phil Trans. R. Soc. London B320, 281-298.
5. Wilson, D.B., Bross, T.E., Hofmann, S.L., and Majerus, P.W. (1984) J. Biol. Chem. 259, 11718-11724.
6. Ryu, S.H., Suh, P.G., Cho, K.S., Lee, K.Y., and Rhee, S.G. (1987) Proc. Natl. Acad. Sci. U.S.A. 84, 6649-6653.
7. Bennett, C.F., and Crooke, S.T. (1987) J. Biol. Chem. 262, 13789-13797.
8. Katan, M., and Parker, P. (1987) Eur. J. Biochem. 168, 413-418.
9. Homma, Y., Imakai, J., Nakanishi, O., and Takenawa, T. (1988) J. Biol. Chem. 263, 6592-6598.
10. Fukui, T., Lutz, R.J., and Lowenstein, J.M. (1988) J. Biol. Chem. 263, 17730-17737.
11. Banno, Y., Yada, Y., and Nozawa, Y. (1988) J. Biol. Chem. 263, 11459-11465.
12. Dawson, R.M.C., Freinkel, N., Jungalwala, F.B., and Clarke, N. (1971) Biochem. J. 122, 605-606.
13. Lapetina, E.G. and Michell, R.H. (1973) Biochem. J. 131, 433-442.
14. Wilson, D.B., Bross, T.E., Sherman, W.R., Berger, R.A., and Majerus, P.W. (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 4013-4017.
15. Koch, M.A., and Diringer, H. (1974) Biochem. Biophys. Res. Commun. 58, 361-367.
16. Ishii, H., Connolly, T.M., Bross, T.E., and Majerus, P.W. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 6397-6401.
17. Sekar, M.C., Dixon, J.F., and Hokin, L.E. (1987) J. Biol. Chem. 262, 340-344.
18. Dixon, J.F., and Hokin, L.E. (1987) J. Biol. Chem. 262, 13892-13895.
19. Dixon, J.F., and Hokin, L.E. (1987) Biochem. Biophys. Res. Commun. 149, 1208-1213.
20. Hughes, A.R., Takemura, H., and Putney, J.W., Jr. (1988) J. Biol. Chem. 263, 10314-10319.

21. Tarver, A.P., King, W.G., and Rittenhouse, S.E. (1987) J. Biol. Chem. 262, 17268–17271.
22. Wong, N.S., Barker, C.J., Shears, S.B., Kirk, C.J., and Michell, R.H. (1988) Biochem. J. 252, 1–5.
23. Downes, C.P., Mussat, M.C., and Michell, R.H. (1982) Biochem. J. 203, 169–177.
24. Connolly, T.M., Bross, T.E., and Majerus, P.W. (1985) J. Biol. Chem. 260, 7868–7874.
25. Irvine, R.F., Letcher, A.J., Heslop, J.P., and Berridge, M.J. (1986) Nature (London) 320, 631–634.
26. Hansen, C.A., Mah, G., and Williamson, J.R. (1986) J. Biol. Chem. 261, 8100–8103.
27. Ryu, S.H., Lee, S.Y., Lee, K.Y., and Rhee, S.G. (1987) FASEB J. 1, 388–393.
28. Connolly, T.M., Bansal, V.S., Bross, T.E., Irvine, R.F., and Majerus, P.W. (1987) J. Biol. Chem. 262, 2146–2149.
29. Hawkins, P.T., Berrie, C.P., Morris, A.J., and Downes, C.P. (1987) Biochem. J. 243, 211–218.
30. Wilson, D.B., Connolly, T.M., Bross, T.E., Majerus, P.W., Sherman, W.R., Tyler, A.N., Rubin, L.J., and Brown, J.E. (1985) J. Biol. Chem. 260, 13496–13501.
31. Irvine, R.F., Letcher, A.J., Lander, D.J., and Berridge, M.J. (1986) Biochem. J. 240, 301–304.
32. Willcocks, A.L., Strupish, J., Irvine, R.F., and Nahorski, S.R. (1989) Biochem. J. 257, 297–300.
33. Lee, C.H., and Hokin, L.E. (1989) Biochem. Biophys. Res. Commun. 159, 561–565.
34. Rhee, S.G., Suh, P.G., Ryu, S.H., and Lee, S.Y. (1989) Science 244, 546–550.
35. Suh, P.G., Ryu, S.H., Moon, K.H., Suh, H.W., and Rhee, S.G. (1988) Cell 54, 161–169.
36. Gerfen, C.R., Choi, W.C., Suh, P.G., and Rhee, S.G. (1988) Proc. Natl. Acad. Sci. U.S.A. 85, 3208–3212.
37. Suh, P.G., Ryu, S.H., Choi, W.C., Lee, K.Y., and Rhee, S.G. (1988) J. Biol. Chem. 263, 14497–14504.
38. Dean, N.M., and Moyer, J.D. (1987) Biochem. J. 242, 361–366.