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DIFFERENT SUSCEPTIBILITY OF ALKYLACYL - VERSUS DIACYL - AND ALKENYLACYL - PHOSPHATIDYLCHOLINE SUBCLASSES TO STIMULATION OF BIOSYNTHESIS BY PHOSPHOLIPASE C

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SUMMARY : Krebs II ascites cells were incubated with [^{3}H] or [^{14}C] choline in the presence or in the absence of Clostridium welchii phospholipase C (PLC). At enzyme concentrations where cell lysis remained limited, PLC specifically enhanced phosphatidylcholine (PC) biosynthesis, as shown by comparison with [^{14}C] ethanolamine. Further analysis revealed that the stimulating effect of PLC remained limited to 1,2-diacyl-sn-glycero-3-phosphocholine (diacyl-GPC) and 1-alkenyl-2-acyl-GPC, whereas the biosynthesis of 1-alkyl-2-acyl-GPC, the putative precursor of platelet activating factor (PAF-acether) remained unchanged. These differences reflect different localizations of the three PC subclasses in the plasma membrane and are discussed in relation to the regulation mechanism of PC biosynthesis.

Using a new procedure of determination, we recently reported a high occurrence of ether-phospholipids (ether-PL) in Krebs II ascites cells, a tumoral cell line maintained in Swiss mice (1). A growing interest for these PL species arose these last years since structural determinations as well as various metabolic and enzymatic studies suggested that 1-alkyl-2-acyl-sn-glycero-3-phosphocholine (alkyl-acyl-GPC) could represent a biochemical precursor to platelet activating factor (1-alkyl-2-acetyl-GPC or PAF-acether), a potent biological mediator active on platelets and leukocytes and able to evoke various pulmonary and cardiovascular effects (2-9).

However, the regulation of the biosynthesis of alkylacyl-,alkenylacyl- and diacyl- GPC has never been investigated in any cell system. Actually, the factors involved in the specific accumulation of ether-phospholipids in certain cells (10-16) are yet poorly understood. Although several studies reported an increased phosphatidylcholine (PC) biosynthesis upon cell stimulation by various agents (17-24), no discrimination was made between the various subclasses.

Abbreviations : PL, phospholipids ; GPC, sn-glycero-3-phosphocholine ; PC, phosphatidylcholine ; PLC, phospholipase C.

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In the present study, Krebs ascites cells have been treated by phospholipase C (PLC) under non-lytic conditions, which promoted an increased synthesis of PC. Analysis of the three PC subclasses revealed that the stimulation was limited to diacyl- and alkenylacyl- GPC, with no effect on PAF-acether precursors. These data are compared to recent findings on membrane sidedness of ether-PL (25,26) and illustrate a possible relationship between phospholipid metabolism and membrane transverse asymmetry.

MATERIAL AND METHODS

[Me- ^{14}C] choline chloride (58 mCi/mmole), [Me- ^{3}H] choline chloride (78 Ci/mmole) and [2-^{14}C] ethanolamine (55 mCi/mmole) were purchased from the Radiochemical Centre, Amersham, UK. Purified PLC from Clostridium welchii (EC 3.1.4.3) was a gift of Prof. RFA Zwaal (27). Minimum Essential Medium of Eagle was supplied by Seromed, Lille, France.

Incubation of cells

Krebs II cells were maintained by weekly intraperitoneal injection into Swiss mice, as already described (28). After harvesting, cells were washed twice in calcium-free Tyrode buffer (pH 6.5) and suspended in Eagle medium buffered to pH 7.4 with 40 mM Hepes (4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid). Final concentration was adjusted to 2 x 10^6 cells/ml, using a Nageotte couting cell.

Cells were then incubated at 37° C for up to 3 h in the presence or in the absence of PLC, after addition of various labelled precursors : 1 µCi/ml of either [^{14}C] or [^{3}H] choline (resulting in specific radioactivities of 41 mCi/mmole and 140 mCi/mmole, respectively, taking in account the concentration of choline in Eagle medium).

Incubations were stopped by rapid centrifugation (600 x g for 2 min). The supernatant was used for lactate dehydrogenase determination (29,30). The pellet received immediately 1 ml of cold 10 mM EDTA and phospholipids were extracted according to Bligh and Dyer (31).

Phospholipid analysis

Various phospholipids were separated on Silicagel G thin layer plates, 0.25 mm thick (Merck, Darmstadt, FRG) using a two dimensional system (32). Separation of diacyl- and ether- PL subclasses was performed after phospholipase A_1 hydrolysis, using a bidimensional thin layer chromatography involving exposure to HCl- fumes between the two runs, as described (1).

Radioactivity was determined in lipid extracts or in the various spots revealed by exposure to iodine vapor, using a Kontron Intertechnique counter (type SL 4000) and Picofluor (Packard, USA) as a scintillation fluid. Phosphorus was measured according to Böttcher et al (33).

RESULTS

Treatment of Krebs II cells with PLC promoted a biphasic effect on [^{14}C] choline incorporation into PL. As shown in Fig. 1, enzyme concentrations inferior to 5 mU/ml increased [^{14}C] choline incorporation in a dose-dependent manner. However, after reaching a maximum at 5 mU/ml, [^{14}C] choline labelling progressively declined. As also indicated in Fig. 1, low phospholipase concen-

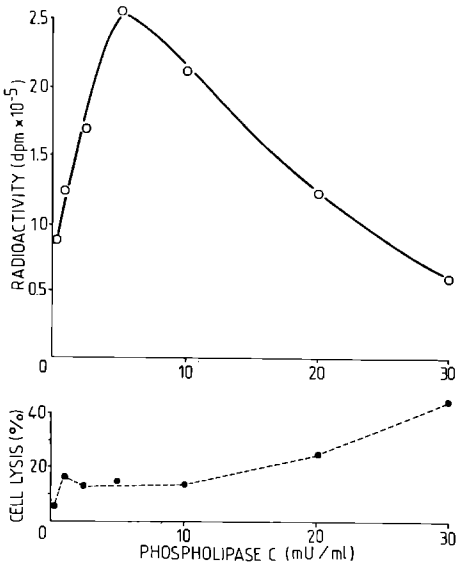


Fig. 1 : Effect of increasing phospholipase C concentrations on [^{14}C] choline incorporation into Krebs II cell phosphatidylcholine and on cell lysis. Cells were incubated for 3 h at 37° C as described under Materials and Methods. [^{14}C] choline incorporation is expressed as dpm/2.10^6 cells, cell lysis corresponds to percentages of total lactate dehydrogenase determined in supernatants.

trations induced a minor cell lysis, whereas as much as 40 % leakage of lactate dehydrogenase was measured in the presence of 40 mU/ml PLC. Thus an enzyme concentration of 5 mU/ml, which allowed a maximal stimulation of PC biosynthesis with a minimal cell lysis, was chosen for further experiments.

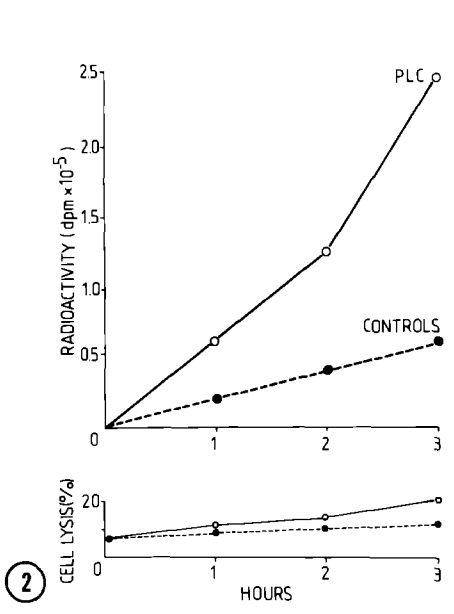
Data from Table 1 indicate that [^{14}C] choline entered essentially PC, sphingomyelin labelling remaining non significant. The effect was specific for choline, since [^{14}C] ethanolamine incorporation was hardly modified by

TABLE 1 Different effect of phospholipase C on [^{14}C] choline and [^{14}C] ethanolamine incorporation into Krebs cell phospholipids

Phospholipase C concentration (mU/ml)	[^{14}C] choline incorporation into		[^{14}C] ethanolamine incorporation into	
	SPH	PC	PC	PE
0	740	32180	590	28510
1			520	20450
2.5			540	22156
5.0	1980	89032	480	22306
10.0			650	25406
20.0			600	23902

Results are expressed in dpm/2.10^6 cells.

SPH, sphingomyelin ; PC, phosphatidylcholine ; PE, phosphatidylethanolamine.



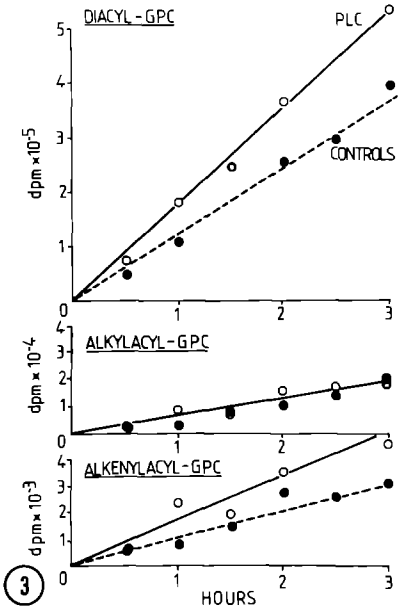


Fig. 2 : Time course of [^{14}C] choline incorporation into Krebs II cell phosphatidylcholine in the presence (○—○) and in the absence (●---●) of phospholipase C. Phospholipase C concentration was 5 mU/ml.

Fig. 3 : Time course of [^{3}H] choline incorporation into the three subclasses of Krebs II cell phosphatidylcholine in the presence (○—○) and in the absence (●---●) of phospholipase C.

PLC treatment. This allows to conclude that PLC had a direct effect on PC biosynthesis rather than a non specific action on membrane permeability for the labelled precursors. Table 1 also indicates that phosphatidylethanolamine methylation was not involved to a significant extent under our experimental conditions, since PC labelling by [^{14}C] ethanolamine remained very low.

Fig. 2 shows a time course of [^{14}C] choline incorporation into Krebs cell phospholipids with or without treatment by 5 mU/ml PLC. The increase in PC labelling promoted by PLC started immediately and could even be observed as soon as 15 min after enzyme addition (not shown). In 11 experiments, it was found that 5 mU PLC stimulated PC biosynthesis by 1.4 to 2.8 fold. Under the same conditions, total cell phospholipid content, as determined by phosphorus measurement, did not vary significantly.

In a series of experiments, the three PC subclasses were separated from each other, according to our published procedure (1). As shown in Fig. 3, [^{3}H] choline incorporation under basal conditions was the highest in diacyl-GPC and the lowest in alkenylacyl-GPC, alkylacyl-GPC displaying an intermediate picture. However, the most striking observation was the lack of PLC effect on alkylacyl-GPC labelling, whereas [^{3}H] choline incorporation into

diacyl- and alkenylacyl- GPC was stimulated by 1.5 and 1.6 fold, respectively. These values reasonably agree with data of Fig. 2 concerning total PC.

DISCUSSION

Sleight and Kent (17,21) have previously shown that PLC enhances PC biosynthesis in several cell lines. Such a treatment induces a specific adsorption of cytosolic CTP : phosphocholine cytidylyltransferase (EC 2.7.7.15) onto microsomal membranes. The subsequent activation of the cytidylyltransferase, which catalyzes the limiting step of PC biosynthesis, leads to an increased production of PC. The same mechanism has been shown to occur during stimulation of PC biosynthesis under various conditions (17-24).

The present study shows that Krebs II ascites cells are responsive to PLC treatment and that the stimulation of PC biosynthesis is absolutely similar to that observed in other cell systems. Indeed, preliminary experiments revealed that CTP : phosphocholine cytidylyltransferase is also in Krebs cells the regulatory enzyme of PC biosynthesis and that PLC treatment promotes its specific adosrption onto microsomal membranes (Tercé et al., in preparation).

Apparently, the increased PC production compensates for PC hydrolysis induced by PLC, this PL being the preferred substrate of the enzyme (27). Indeed, no change in PL content could be detected in cells over 3 h PLC treatment.

The advantage of using Krebs II cells in this experimental model is that the relatively high amount of ether-PL should allow to examine the behaviour of the three PC subclasses, which was never previously emphasized. Actually, our study revealed a striking difference between the three subclasses, since only diacyl- and alkenylacyl-GPC were susceptible to PLC action, alkylacyl-GPC remaining refractory to the stimulation evoked by the enzyme. We recently found that PL subclasses display a highly asymmetric distribution through the plasma membrane of Krebs cells (25,26). For instance, diacyl-GPC and alkenylacyl-GPC are almost entirely located in the outer leaflet of the membrane, whereas alkylacyl-GPC remains specifically confined to the inner leaflet. It is thus tempting to conclude that PLC effect was limited to those PC subclasses directly exposed on the cell surface. This would suggest that PC resynthesis involves the diglycerides generated by PLC action. Indeed, diglycerides can easily cross the membrane bilayer (34,35). Such a reutilization of the diglyceride backbone is thus similar to that reported during the increased turnover of inositol-PL brought about by numerous physiological agonists (36).

In conclusion, our study brings a new insight into the process regulating PC biosynthesis. In the last step of PC building, besides the availability of the hydrosoluble substrate, CDP-choline, the amount and the nature

of the lipophilic substrate diacylglycerol might also represent a critical parameter. This could give a key in understanding the mechanism by which a cell regulates its relative content of diacyl- and ether- PL. Such a process is specially important in the case of alkylacyl-GPC, owing to its role in PAF-acether biosynthesis and should justify further studies using the experimental model depicted herein.

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REFERENCES

1. El Tamer, A., Record, M., Fauvel, J., Chap, H. and Douste-Blazy, L. (1984) Biochim. Biophys. Acta 793, 213-220.
2. Demopoulos, C., Pinckard, R.N. and Hanahan, D.J., (1979) J. Biol. Chem. 254, 9355-9358.
3. Benveniste, J., Tercé, M., Varenne, P., Bidault, J., Boullet, C. and Polonsky, J. (1979) C.R. Acad. Sci. Ser. D 289, 1037-1040.
4. Blank, M.L., Snyder, F., Byers, L.W., Brooks, B. and Muirhead, E.E. (1979) Biochem. Biophys. Res. Commun. 90, 1194-1200.
5. Hanahan, D.J., Demopoulos, C.A., Liehr, J. and Pinckard, R.N. (1980) J. Biol. Chem. 255, 5514-5516.
6. Chap, H., Mauco, G., Simon, M.F., Benveniste, J. and Douste-Blazy, L. (1981) Nature 289, 312-314.
7. Wykle, R.L., Malone, B. and Snyder, F. (1980) J. Biol. Chem. 255, 10256-10260.
8. Alonso, F., Gil, M.G., Sanchez-Crespo, M. and Mato, J.M. (1982) J. Biol. Chem. 257, 3376-3378.
9. Ninio, E., Mencia-Huerta, J.M., Heymans, F. and Benveniste, J. (1982) Biochim. Biophys. Acta 710, 23-31.
10. Horrocks, L.A. (1968) in Ether Lipids, Snyder, F., ed., pp. 177-262, Academic Press, New York.
11. Horrocks, L.A. (1982) in Phospholipids, Hawthorne, J.N. and Ansell, G.B., eds, pp 51-93, Elsevier, Amsterdam.
12. Diagne, A., Fauvel, J., Record, M., Chap, H. and Douste-Blazy, L. (1984) Biochim. Biophys. Acta 793, 221-231.
13. Mueller, H.W., O'Flaherty, J.T. and Wykle, R.L. (1982) Lipids 17, 72-77.
14. Sugiura, T., Onuma, Y., Sekiguchi, N. and Waku, K. (1982) Biochim. Biophys. Acta 712, 515-522.
15. Sugiura, T., Nakajima, M., Sekiguchi, N., Nakagawa, Y. and Waku, K. (1983) Lipids 18, 125-129.
16. Natarajan, V., Zuzarte-Augustin, M., Schmid, H.H.O. and Graff, G. (1982) Thromb. Res. 30, 119-125.
17. Sleight, R. and Kent, C. (1980) J. Biol. Chem. 255, 10644-10650.
18. Weinhold, P.A., Feldman, D.A., Quade, M.M., Miller, J.C. and Brooks, R.L. (1981) Biochim. Biophys. Acta 665, 134-144.
19. Pritchard, P.H., Chiang, P.K., Cantoni, G.L. and Vance, D.E. (1982) J. Biol. Chem. 257, 6362-6367.
20. Limp, P.H., Pritchard, P.H., Paddon, H.B. and Vance, D.E. (1983) Biochim. Biophys. Acta 753, 74-82.
21. Sleight, R. and Kent, C. (1983) J. Biol. Chem. 258, 824-829.
22. Sleight, R. and Kent, C. (1983) J. Biol. Chem. 258, 831-835.
23. Pelech, S.L., Pritchard, P.H., Brindley, D.N. and Vance, D.E. (1983) J. Biol. Chem. 258, 6782-6788.

24. Pelech, S.L., Power, E. and Vance, D.E. (1983) Can. J. Biochem. Cell Biol. 61, 1147-1152.
25. Record, M., El Tamer, A., Diagne, A., Fauvel, J., Chap, H. and Douste-Blazy, L. (1983) in Platelet Activating Factor, INSERM Symposium N° 23, Benveniste, J. and Arnoux, B., eds, Elsevier, Amsterdam, pp. 283-290.
26. Record, M., El Tamer, A., Chap, H. and Douste-Blazy, L. (1984) Biochim. Biophys. Acta, in press.
27. Zwaal, R.F.A., Roelofsen, B. Comfurius, P. and van Deenen, L.L.M. (1975) Biochim. Biophys. Acta 406, 83-96.
28. Record, M., Bes, J.C., Chap, H. and Douste-Blazy, L. (1982) Biochim. Biophys. Acta 688, 57-65.
29. Chap, H., Zwaal, R.F.A. and van Deenen, L.L.M. (1977) Biochim. Biophys. Acta 467, 146-164.
30. Wroblewski, F. and La Due, J.S. (1955) Proc. Soc. Exp. Biol. 90, 210-215.
31. Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911-918.
32. Esko, J.D. and Raetz, C.R.H. (1980) J. Biol. Chem. 255, 4474-4480.
33. Böttcher, C.J.F., van Gent, C.M. and Pries, C. (1961) Anal. Clin. Acta 24, 203-204.
34. Allan, D., Low, M.G., Finean, J.B. and Michell, R.H. (1975) Biochim. Biophys. Acta 413, 309-316.
35. Mauco, G., Chap, H., Simon, M.F. and Douste-Blazy, L. (1978) Biochimie 60, 653-661.
36. Michell, R.H. (1975) Biochim. Biophys. Acta 415, 81-147.