NMR Studies of Phospholipase C Hydrolysis of Phosphatidylcholine in Model Membranes

Shastri P. Bhamidipati and James A. Hamilton

From the Department of Biophysics, Housman Medical Research Center, Boston University School of Medicine, Boston, Massachusetts 02118-2394

Hydrolysis of phospholipids in biological membranes by phospholipase C (PLC) produces an important second messenger molecule, 1,2-diacylglycerol (DAG), that is essential for the activation of protein kinase C (PKC). While the effects of DAG on model membranes have been investigated earlier, studies on physical properties of DAG introduced into phospholipid bilayers by PLC have been lacking. We present an NMR approach for studying structural and kinetic aspects of PLC-mediated hydrolysis of 13 carbonyl-enriched phospholipids in model membranes (small unilamellar vesicles). The product DAG is readily detected by 13C NMR, and its structural properties as well as those of the model membrane can be monitored continuously. PLC hydrolysis was limited to a low proportion of the model membrane by incorporating a small amount of ester phospholipid into a nonhydrolyzable ether-linked phospholipid matrix. Under these conditions, PLC (Bacillus cereus) hydrolyzed only the monolayer of phosphatidylcholine to which it was exposed (the outer monolayer). The 1,2-DAG product remained associated with the membrane bilayer and did not alter bilayer structure in any detectable way. From the chemical shift data, it is inferred that the DAG has an interfacial conformation similar to that of phosphatidylcholine. These results show that DAG could activate PKC by direct interaction with the enzyme rather than by perturbation of the membrane bilayer.

The phospholipase C (PLC) reaction is a key intermediate step in signal transduction across cell membranes (Pelech and Vance, 1989; Exton, 1990; Bell and Burns, 1991; Merrill and Liotta, 1991). Extracellular agonists such as hormones and growth factors bind to cell receptors, triggering PLC-mediated hydrolysis of the membrane constituents phosphatidylcholine (PC) and phosphatidylinositol to produce the important second messenger, 1,2-diacylglycerol (DAG). The replacement of a choline- or inositolphosphate group by a hydroxyl group results in a molecular structure which activates protein kinase C (PKC). The phosphorylating activity of PKC then produces cellular responses such as contraction, secretion, and cell proliferation.

Although the molecular details of PKC activation are incomplete, activation occurs in the membrane and the sn-1,2 configuration of DAG is essential (Nishizuka, 1984; Bell and Burns, 1991; Merrill and Liotta, 1991). There is considerable interest in whether DAG exerts its influence by acting within the plane of a stable bilayer membrane or by disrupting the membrane bilayer structure (Epand and Lester, 1990; Zidovetski and Lester, 1992). The latter possibility has been suggested by studies showing that DAG can promote bilayer to hexagonal phase changes in model membranes (Epand and Lester, 1990; Siegel et al., 1989), while the former is plausible because significant amounts of DAG can be incorporated into the bilayer structure of phospholipids such as PC (de Boeck and Zidovetski, 1989; Hamilton et al., 1991a), the most common phospholipid in membranes. However, model systems to date have not examined the properties of DAG introduced into membranes by PLC action.

Previous studies of PLC action on cell membranes and model membranes have been aimed primarily at determining whether the enzyme can be used to probe the asymmetric distribution of phospholipids. Inconsistent results were obtained for PLC from the same source (Bacillus cereus) acting on different cell membranes (Demant et al., 1979; Goldfine et al., 1982). In model membrane systems, PLC activity appeared to be restricted to the leaflet to which it was exposed only when the mole fraction of hydrolyzable lipids in the vesicle was kept low (≤0.2) (Sundler et al., 1978). It was suggested that when the mole fraction of hydrolyzable lipid is higher (>0.2), the DAG produced may not orient at the interface of the bilayer but partition into the hydrocarbon interior and alter the bilayer structure, allowing the enzyme to hydrolyze both leaflets.

The present study describes a new approach to monitor the PLC reaction in model membranes. The NMR methods described are uniquely suited to determine: (i) whether the enzyme hydrolyzes only one leaflet of the bilayer selectively, (ii) where the products are located in the membrane, and (iii) whether the bilayer structure is altered during hydrolysis.

Previous 13C NMR studies of DAG in uni- and multilamellar PC bilayers (Hamilton et al., 1991a, 1991b) provide essential background data for the present study, which represents a more biologically relevant way of introducing DAG into membranes, by PLC hydrolysis of phospholipid bilayers.

Our strategy is described as follows, with reference to the schematized reaction of PC hydrolysis by PLC (Fig. 1). 13C enrichment of one or both carbonyls of PC was used to improve the detectability of the carbonyl signal for 13C NMR analysis. The carbonyl chemical shift is sensitive to intrinsic factors (chemical bonding and structure), and well-resolved signals can be observed for substrate (PC) and product (DAG) in a model membrane (Hamilton et al., 1991a). Moreover, the
Fig. 1. Schematic representation of PLC reaction on 1,2-diacyl phosphatidylcholine showing the \textsuperscript{13C} enrichment positions of the phospholipid with sn-1 in 	extit{parentheses}. The scheme also emphasizes the naturally 100% abundant \textsuperscript{31}P nucleus in the substrate PC and the product, choline phosphate. All experiments presented here, except for one, used PC with enrichment in the sn-2-carbonyl alone.

\textsuperscript{13}C carbonyl chemical shift is highly sensitive to extrinsic factors, such as hydrogen bonding and other local interactions, and provides information about the molecular organization and conformation of lipids in bilayers (Hamilton et al., 1991a, 1991b). In order to model the normal biological action of PLC, which is limited to a small percentage of phospholipid in a membrane (Pellech and Vance, 1989; Exton, 1990; Bell and Burne, 1991; Merrill and Liootta, 1991), low proportions of the \textsuperscript{13}C-labeled PC were incorporated into a nonhydrolyzable ether phospholipid bilayer. Ether phospholipids have earlier been used to study the mechanistic aspects of lipolytic enzymes (Burns et al., 1981; Massey et al., 1985; Pownall et al., 1985a, 1985b; Bhamidipati and Hamilton, 1989). The physical properties of the ether and ester phospholipids have been shown to be very similar by several physicochemical criteria, such as gel-liquid crystal phase transition, miscibility with ester phospholipids, vesicle fusion, and the kinetics of protein-lipid association (McKeon et al., 1986). Besides limiting the extent of hydrolysis in the bilayer, the ether-PC does not produce any signals in the carbonyl region of the spectrum that would interfere with ester-PC signals. Choline phosphate, the second product of PC hydrolysis by PLC, can be detected by \textsuperscript{31}P NMR, and its chemical shift is well-separated from that for the substrate, PC. \textsuperscript{31}P NMR chemical shifts and lineshapes also provide direct information about the bilayer structure of phospholipids (Berden et al., 1974).

EXPERIMENTAL PROCEDURES

Materials—Dihexadecyl PC (DHPC) was obtained from Serdary Research Laboratories, Ontario, Canada. Phospholipase C (\textit{B. cereus}) was from Sigma. \textsuperscript{[13]C}Carbonyl-enriched phospholipids used in this study, namely sn-2-labeled \textsuperscript{[13]C]DPPC, sn-1,2-labeled \textsuperscript{[13]C]DPPC, and sn-2-labeled \textsuperscript{[13]C]}palmitoyl 2-oleoyl PC (POPC), were synthesized as reported earlier (Bhamidipati and Hamilton, 1989). Ditetradeyl PC (DTPC) was provided by Dr. R. N. A. H. Lewis, McMaster University, Hamilton, Ontario, and was synthesized according to published procedures (George et al., 1990).

Preparation of Vesicles—Small unilamellar vesicles (SUVs) were prepared by sonication of appropriate amounts of dried lipid film dispersed in 10 mM Tris buffer (pH 7.4) as before (Bhamidipati and Hamilton, 1989) at temperatures 10 °C above the gel to liquid crystalline phase transition temperature (T\textsubscript{c}) of the ether phospholipid. DPPC (T\textsubscript{c} = 39 °C) was shown earlier to mix ideally with DTPC (T\textsubscript{c} = 26 °C) and DHPC (T\textsubscript{c} = 41 °C) at all compositions (McKeone et al., 1986). Typical concentrations of the total phospholipids were 25-30 \textmu mol/ml.

Chemical Methods—All vesicle samples were analyzed by TLC for lipid integrity. Total phospholipid was quantitated by the Bartlett Method (Bartlett, 1959). Following PLC-mediated hydrolysis, samples were Folch-extracted to quantitate the unreacted phospholipid and to determine the extent of hydrolysis. DAG produced during NMR kinetic experiments were analyzed by TLC for acyl chain migration (Hamilton et al., 1991a).

NMR Measurements—\textsuperscript{13}C NMR spectra were recorded at 50.3 MHz (4.7 tesla) on a Bruker WP-200 NMR spectrometer equipped with an Aspect 2000A data system and a Bruker B-VT-1000 variable-temperature unit. Broad-band proton decoupling (1.0 watt) centered at 3.4 ppm downfield from tetramethylsilane was used. HH\textsubscript{2}O was used as an internal lock and shim signal. The terminal methyl peak of the lipid fatty acyl chains at 14.10 ppm was used as a chemical shift reference (Hamilton et al., 1974); 1 \mu g of the enzyme PLC in the same buffer used for the substrate vesicles (see above) was added to each sample. Samples were maintained at 10 °C above the T\textsubscript{c} of the ether-PC during addition of the enzyme and were transferred directly to the NMR probe pre-equilibrated at the desired temperature. Spectra were obtained as a function of time at 38 °C for DTPC vesicles and 48 °C for DHPC vesicles. In a typical kinetic experiment, spectra were obtained every 0.6 h for the first 6 h and every 1.2 h for the next 6 h.

\textsuperscript{31}P NMR spectra were obtained at 81.0 MHz with broadband proton decoupling on the same NMR instrument as described above. Chemical shifts of the phospholipid signals were measured with reference to external phosphoric acid. Spectra were accumulated with a 5000-Hz spectral width, 8192 time domain points, a 2.5-s pulse interval, and 300 scans.

Light Microscopy—Phospholipid samples of known composition (see "Results" and "Discussion") were examined by direct and polarized light with a polarizing microscope (Leitz Dialux, Wetzlar, Germany) fitted with a heating/cooling stage (Model 80, Leitz) and a thermocouple/digital thermometer (Doric Model 450, San Diego, CA). Changes in optical texture were observed during heating and cooling scans (1 °C/min) in the temperature range 30-60 °C. Dried lipid samples were uniformly spread on microscope slide with the aid of a coverslip, and a small volume of buffer (50 \mu l) was added to the edge of the coverslip to observe hydration of the lipid film.

RESULTS AND DISCUSSION

Fig. 2 illustrates the use of \textsuperscript{13}C NMR to monitor PLC-mediated hydrolysis of SUVs comprised of 10 mol % sn-2-labeled \textsuperscript{[13]C]DPPC and 90 mol % DTPC at 38 °C. The spectrum of the substrate vesicles contained two signals in the

**Fig. 2.** 50.3 MHz \textsuperscript{13}C NMR spectra (carbonyl region) of 10 mol % sn-2-labeled \textsuperscript{[13]C]DPPC/90 mol % DTPC vesicles in 10 mM Tris buffer at 38 °C. The panel on the left shows the substrate (top spectrum) before addition of the enzyme and selected spectra corresponding to indicated time points, following addition of 1 \mu g of PLC; All spectra were obtained under identical conditions (1000 accumulations, 2.0-s pulse interval, and 16,384 size) and processed using 2-Hz line broadening and absolute intensity scaling. The panel on the right shows difference spectra obtained by subtracting the substrate spectrum from the spectrum for each time interval as indicated. Insert shows the rate of hydrolysis as determined from the carbonyl peak area of the substrate.
These signals reflect only the hydrolyzable component of the expected -211 ratio for PC in the outer and inner leaflets of bilayer (13C-enriched DPPC) since DTPC lacks the ester carbonyl groups. The relative peak intensities show the expected ~2/1 ratio for PC in the outer and inner leaflets of SUVs (Hamilton and Small, 1981). Phospholipase C (B. cereus) was added in buffer to the aqueous solution containing vesicles, and the hydrolytic reaction was followed as a function of time (Fig. 2, left panel). The 13C(carbonyl) spectrum showed a progressive decrease in the signal for DPPC in the outer monolayer of the vesicle (P), the leaflet to which the enzyme is exposed. The signal for DPPC in the inner monolayer of vesicles (P) was unaffected throughout hydrolysis, indicating no exposure of the inner leaflet DPPC to PLC, either by penetration of the enzyme or by movement of DPPC from the inner to the outer leaflet. A new signal at 173.35 ppm appeared during the first time interval (0.6 h), and its intensity relative to the PC carbonyl peaks increased with time. This signal corresponds to the newly formed DAG, i.e. sn-2-labeled [13C]DPPC. This chemical shift is the same as that observed for various DAGs incorporated in SUVs comprised of ester-PC and reflects the association of DPG with the phospholipid bilayer (Hamilton et al., 1991a, 1991b). The single peak observed for DPPC does not differentiate its distribution in the two leaflets of the bilayer, although it can undergo rapid (millisecond) flip-flop in vesicles. Difference spectra (Fig. 2, right panel) show a quantitative correspondence between the gain in DPG signal and the loss of DPPC signal from the outer monolayer. These spectra show no intensity for DPPC signal from the inner monolayer, which did not change during hydrolysis.

The rate and extent of hydrolysis of DPPC by PLC was estimated from the time-dependent decrease in the PC carbonyl peak area (Fig. 2, inset). At the end of 3.5 h of PLC treatment, 35% of the total [13C]DPPC was hydrolyzed, an estimate verified by chemical analysis. This corresponds to 50% of the PC in the outer monolayer and represents only 3.5 mol % of the total PC (ester and ether PC). The specific activity of PLC for the DPPC/DTDPC vesicles was calculated as 11.0 amol min⁻¹ mg⁻¹, similar to that previously reported for DPPC/DHPC vesicles (El-Sayed et al., 1985). This slow rate of hydrolysis is appropriate for the 13C NMR studies, which required signal averaging for 30 min to obtain suitable signal-to-noise ratios.

The chemical shift of 1,2-DAG in vesicles is distinct from that of 1,3-isomer, which can form spontaneously from the 1,2-isomer (Hamilton et al., 1991a; Kodali et al., 1990). Thin-layer chromatography analysis of DAG from the product complex following the NMR experiments detected only the 1,2-isomer.

In egg-PC vesicles, such a distinction was seen only at high proportions of DAG with respect to PC (≥15 mol %) and at temperatures <38 °C (Hamilton et al., 1991a).

Fig. 3. 50.3 MHz carbonyl region 13C NMR spectra of: A, 10 mol % sn-2-labeled [13C]DPPC; B, 10 mol % sn-1,2-labeled [13C]DPPC; and C, 10 mol % sn-2-labeled [13C]POPC in DHPC SUVs following 3 h of hydrolysis by PLC at 48 °C. All spectra were obtained and processed the same way as mentioned in Fig. 2. P, represent the outer and the inner monolayer phospholipids.

The chemical shift of 1,2-DAG in vesicles is distinct from that of the 1,3-isomer, which can form spontaneously from the 1,2-isomer (Hamilton et al., 1991a; Kodali et al., 1990). Thin-layer chromatography analysis of DAG from the product complex following the NMR experiments detected only the 1,2-isomer.

In egg-PC vesicles, such a distinction was seen only at high proportions of DAG with respect to PC (≥15 mol %) and at temperatures <38 °C (Hamilton et al., 1991a).
PLC treatment. The chemical shift separation and the relative intensities of the two signals for substrate vesicles are typical of phospholipids present in SUVs and correspond to outer and inner monolayer phospholipid. The bilayer structure of the vesicles was preserved following a 6-h treatment by PLC, as revealed by the 31P NMR spectrum of the product complex (Fig. 4, bottom spectrum). The 13C carbonyl spectrum showed signals for PC on both leaflets throughout hydrolysis (Figs. 2 and 3) verifying that the ester-linked PC component was present as a bilayer. Thus, the DAG produced did not induce any gross changes in the structural organization of the bilayer. The 31P NMR spectrum also shows a very weak signal for choline phosphate, consistent with hydrolysis of a relatively small amount of the total phospholipid. We conclude that ether-linked PC was not hydrolyzed, as expected (El-Sayed et al., 1985).

Since neither 13C nor 31P NMR spectra can rule out the possibility of DAG-induced phase changes in a minor proportion of the ester- or ether-linked PC, polarizing light microscopy was performed on three different samples of the following composition: (i) 10 mol % DPPC, 90 mol % DHPC, (ii) 5 mol % 1,2-DPG, 5 mol % DPPC, 90 mol % DHPC, and (iii) 10 mol % 1,2-DPG, 90 mol % DHPC. A uniformly spread dried lipid film of each composition was hydrated with buffer and monitored under direct and polarizing light (see "Experiments Procedures") from 30–60°C. All the three samples showed optical textures typical of lamellar structure (myelin figures) with no evidence of any hexagonal phase.

In conclusion, 13C and 31P NMR provide simple procedures for following the PLC hydrolysis of phospholipid bilayers in a time-dependent fashion and avert the need to extract lipids for quantitation of the reaction, which would result in loss of structural organization of the membrane. The procedure also monitors the properties of the product DAG continuously. Under conditions of limited hydrolysis, the NMR results showed that: (i) the bilayer structure of the vesicles remained intact, (ii) the enzyme cleaved only the PC on the outer leaflet of the vesicles, and (iii) the product DAG fits into the bilayer structure of the model membrane with an interfacial conformation similar to that of PC. The properties of DAG produced by PLC in model membranes described above are identical to those for model systems prepared by hydration of mixed lipid films (Hamilton et al., 1991a). While our results showed no fundamental changes in membrane structure, some undetected but significant changes that can occur in the membrane as a result of limited production of DAG are: (i) a decrease in charge density, if the charged headgroup diffuses away from the site of production, and (ii) a rapid diffusion (flip-flop) of DAG across the bilayer, since the barrier to spontaneous translocation (polar headgroup) across the lipid bilayer has been removed (Hamilton et al., 1991a).

The above findings suggest that PKC may interact with DAG in an undisrupted bilayer. The levels of DAG produced in these model membranes are likely sufficient for their role as second messenger in vivo. In phospholipid SUVs containing 1–10 mol % 1,2-DAG, both autophosphorylation and substrate phosphorylation activities of PKC are stimulated (Newton and Koshland, 1990). The specific model membranes used in this study lack essential cofactors required for activation of PKC (i.e. PS and Ca2+), but the molecular conformation of DAG is the same in mixed ester-PC and ether-PC model membranes as in ester-PC vesicles containing PS and Ca2+

Since PLC produces a potent activator very similar to the inactive parent phospholipid in molecular conformation and chemical structure, except for the polar headgroup, discrimination of these two closely related molecules by PKC is likely to occur at the membrane interface.

Acknowledgments—We thank Dr. R. N. A. H. Lewis, McMaster University, Hamilton, Ontario for his generous gift of DTPC and acknowledge Margaret Gibbons for preparation of the manuscript.

REFERENCES
Bartlett, G. R. (1959) J. Biol. Chem. 234, 466–468
Bell, R. M. and Burns, D. J. (1991) J. Biol. Chem. 266, 4681–4684
Berden, J. A., Cullis, P. R., Houtl, D. L., McLaughlin, A. C., Radda, G. K. and Richards, R. E. (1974) FEBS Lett. 46, 55–58
Bhamidipati, S. P. and Hamilton, J. A. (1989) Biochemistry 28, 6667–6672
Burns, R. A., Jr., Friedman, J. M. and Roberts, M. F. (1981) Biochemistry 20, 5645–5650
de Boeck, H. and Zidovetski, R. (1989) Biochemistry 28, 7439-7446
Demant, E. J. F., Op Den Kamp, J. A. F. and Van Deenen, L. L. M. (1979) Eur. J. Biochem. 95, 613–617
El-Sayed, M. M., DelBello, C. D., Coeuy L. A. and Roberts, M. F. (1985) Biochim. Biophys. Acta 837, 320–333
Epand, R. M., and Lester, D. S. (1980) Trends Pharmacol. Sci. 11, 317–320
Exton, J. H. (1991) J. Biol. Chem. 265, 1–4
George, R., Lewis, R. N. A. and McElhaney, R. N. (1990) Biochim. Biophys. Acta 1060, 161–169
Goldfine, H., Johnson, N. C. and D’Souza, D. G. (1982) Biochem. Biophys. Res. Commun. 105, 1502–1507
Hamilton, J. A., Tolkowski, C., Childers, R. T., Williams, A., Allerhand, A., and Cordes, E. H. (1974) J. Biol. Chem. 249, 4872–4878
Hamilton, J. A., and Small, D. M. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 6878–6882
Hamilton, J. A., Bhamidipati, S. P., Kodali, D. R., and Small, D. M. (1991a) J. Biol. Chem. 266, 1177–1186
Hamilton, J. A., Fujito, D. T., and Hammer, C. F. (1991b) Biochemistry 30, 2884–2892
Kodali, D. K., Tercyak, A., Fahey, D. A., and Small, D. M. (1990) Chem. Phys. Lipids 52, 163–170
Massey, J. B., Pao, Q., Dunn, S., and Pownall, H. J. (1985) J. Biol. Chem. 260, 11719–11723
McKeone, B. J., Pownall, H. J., and Massey, J. B. (1986) Biochemistry 25, 7211–7216
Merrill, A. J., Jr. and Liotta, D. C. (1991) Curr. Opin. Struct. Biol. 1, 516–521
Newton, A. C., and Koshland, D. E. Jr. (1990) Biochemistry 29, 6656–6661
Nishizuka, Y. (1984) Nature 308, 655–655
PelcB, S. L. and Vance, D. E. (1989) Trends Biochem. Sci. 14, 28–30
Pownall, H. J., Pao, Q., Jr., and Massey, J. B. (1983a) J. Biol. Chem. 260, 2146–2152
Pownall, H. J., Pao, Q., and Massey, J. B. (1985b) Biochim. Biophys. Acta 833, 456–462
Siegel, D. P., Banachbch, J., and Yeagle, P. L. (1989) Biochemistry 28, 5010–5014
Smith, S. O., Kustanovich, I., Bhamidipati, S., Salmon, A., and Hamilton, J. A. (1992) Biochemistry 31, 11663–11664
Sundell, R., Alberts, A. W., and Vagelos, P. R. (1978) J. Biol. Chem. 253, 2929–2934
Zidovetski, R., and Lester, D. S. (1992) Biochim. Biophys. Acta 1134, 261–272