Validation of a Simple Radiochemical Assay Measuring Hydrolysis of Choline-Labelled Microsomal Phosphatidylcholine by Phospholipase C

pH-DEPENDENCE

By BRYAN R. CATER,* PAMILLA TRIVEDI and TERENCE HALLINAN
Department of Biochemistry and Chemistry, Royal Free Hospital School of Medicine,
8 Hunter Street, London WC1N 1BP, U.K.

(Received 7 June 1976)

Selective hydrolysis of phosphatidylcholine species, which are selectively radioactively labelled *in vivo*, does not appear to interfere with a radiochemical assay for hydrolysis of microsomal phosphatidylcholine by C-type phospholipases from *Bacillus cereus* or *Clostridium perfringens*. Both phospholipases substantially hydrolysed phosphatidylcholine over the pH range 4.0–10.0.

Phospholipid-dependence or constraint of membrane-bound enzymes can be directly studied by using C-type phospholipases, since these completely destroy diacylphosphatides, unlike other phospholipases whose products are still phospholipids, and in the case of A-type phospholipases are also highly membranolytic. Phospholipase C, used at 0–5°C, also avoids inactivating enzymes that become abnormally temperature-sensitive at physiological temperatures when delipidated (Hallinan, 1974), though the possibility of cold-inactivation must, of course, be excluded before this approach can be used.

Phospholipase C action on membrane phospholipids is conveniently monitored radiochemically if phospholipids are biosynthetically labelled, e.g. with radioactive choline. However, though such radiochemical assays are frequently used (e.g. review by Grossman *et al.*, 1974; see also Hallinan *et al.*, 1969; Lumper *et al.*, 1969; Akao & Omura, 1972; Möllby *et al.*, 1974), there seems to be no evidence that they quantitatively measure phosphatidylcholine hydrolysis. Serious interference could occur from selective hydrolysis of individual phosphatidylcholine species, since all biosynthetic routes label different phosphatidylcholines quite non-randomly in their choline component (reviewed by van den Bosch, 1974). The present paper validates a radiochemical assay for phospholipase C acting on microsomal phosphatidylcholine and briefly re-examines the pH-dependence of C-type phospholipases from *Clostridium perfringens* (*Clostridium welchii*) and from *Bacillus cereus*.

Materials and Methods

Preparation of liver microsomal fractions from male rats, labelled *in vivo* with radioactive choline or ethanolamine, and assays for protein and P_i are

* Present address: BIBRA, Woodmansterne Road, Carshalton, Surrey, U.K.

described by Cater *et al.* (1975). Labelling with [$Me-^{14}C$]methionine was for 60 min *in vivo*, when virtually the only lipid labelled is phosphatidylcholine (Bjornstad, 1966). To obviate correction for ^{14}C spillover in double-label experiments it was arranged that 10–20 times as much 3H as ^{14}C was incorporated into phospholipids. Impure *Cl. perfringens* (*Cl. welchii*) phospholipase C (nominal specific activity 5 units/mg of protein) was purchased from Sigma (London) Chemical Co., London S.W.6, U.K. Specifications of the *B. cereus* phospholipase C, which was purchased from Makor Chemical Co., P.O. Box 6570, Jerusalem, Israel, indicated that it was 8–20 times more active per mg of protein and that it was almost proteinase-free. The specificity of these enzymes against microsomal phospholipids is described in Cater *et al.* (1975). Conditions for treatment with *B. cereus* and *Cl. perfringens* phospholipase C (at pH 6.8 in all experiments in Fig. 1) are also described by Cater *et al.* (1975). Incubation times and temperatures and amounts of phospholipase used are indicated in individual Figure legends.

Assays of phospholipase C action

Hydrolysis of phosphatidylcholine, labelled *in vivo* with [$Me-^{14}C$]methionine, was determined by measuring disappearance of radioactivity from purified lipid extracts (Folch *et al.*, 1957). However, when radioactive choline was used to label phospholipids, after 30–45 min *in vivo* as shown by Nagley & Hallinan (1968), or *in vitro* (P. Trivedi & T. Hallinan, unpublished work), phosphatidylcholine was very selectively labelled, and it accounted for more than 97% of the acid-insoluble radioactivity of microsomal fractions, even when non-radioactive casein hydrolysate was not administered (cf. Nagley & Hallinan, 1968). Others have also reported almost specific incorporation of radioactive choline into liver phosphatidylcholine *in vivo* (e.g. Stein & Stein, 1969;

Cope & Williams, 1969; Young *et al.*, 1971). With such selective incorporation, hydrolysis of phosphatidylcholine labelled by radioactive choline could be very simply followed by using a slight adaptation of a glass-fibre-disc precipitation method (Nagley & Hallinan, 1968; Hallinan *et al.*, 1969). In this method replicate 50 or 100 μ l samples of control or phospholipase C-treated microsomal fractions were plated on to separate 2.1 cm glass-fibre discs (Whatman GF/C or GF/A). No increase in quenching was seen when 50–1550 μ g of protein from [3 H]- or [14 C]-choline-labelled microsomal fractions was plated. Discs were then washed four to six times in 10–30 ml of 0.2M-HClO₄ in small beakers, which removes both acid-soluble radioactive biosynthetic precursors and also the radioactive phosphorylcholine formed by phospholipase C action, whereas intact phospholipids remain on the discs because they are acid-insoluble. Washed discs were then left for 3–5 min in a sealed tank, saturated with vapour from conc. NH₃ (sp.gr. 0.880). This neutralized the HClO₄ and prevented charring when discs were dried under an i.r. lamp. If a secondary scintillant is used during radioactive counting, it also prevents formation of an intense yellow colour, which otherwise seriously interfered with measurements of radioactivity in a Packard 3385 liquid-scintillation spectrometer.

Under several conditions, the glass-fibre-disc assay proved inapplicable for measuring hydrolysis of phospholipids in microsomal fractions.

(i) At 37°C and pH 4.0 or 5.0, substantial radioactivity was lost from microsomal fractions in the absence of phospholipase C. Apparently vesicles aggregated on to the walls of glass tubes in a time-dependent fashion, interfering with discrete sampling techniques. This did not occur at 5°C and was distinguished from phospholipid hydrolysis by extracting the entire contents of incubation tubes and counting radioactivity in purified lipid extracts (Folch *et al.*, 1957). Concentrations of Ca²⁺ higher than 1 mM, which were too high for optimal phospholipase C action on microsomal fractions, and concentrations of cetyltrimethylammonium bromide higher than 0.03%, also tended to interfere by causing microsomal aggregation.

(ii) Radioactivity was also lost from microsomal fractions in the absence of phospholipase C when acid-soluble neutral detergents such as Triton X-100 were present. These seemed to impair acid precipitation of vesicles on discs.

(iii) Digitonin, at a concentration of 0.6%, inhibited phospholipase C by more than 50% (C. Berry, unpublished work).

Assessment of pH effects

In an attempt to minimize buffer effects, pH-dependence was examined in three different composite buffer mixtures, contributing the following

final concentrations of each component to the phospholipase assays: I, Buffer W, an Na⁺-free buffer, 0.005M-acetate, -histidine, -Hepes [2-(N-2-hydroxyethyl)piperazin-N'-yl]ethanesulphonic acid] and -Tris: pH was adjusted with Tris base; II, Buffer X, 0.008M-acetate, -cacodylate, -maleate, -Tris and -borate: pH was adjusted with NaOH; III, Buffer Y, 0.008M-glutamate, -malate, -histidine, -ethylenediamine and -glycylglycine: pH was adjusted with NaOH.

Results and Discussion

Because it is the major component, constituting about 60% of the liver microsomal phospholipids (reviewed by White, 1973), phosphatidylcholine is the individual phospholipid class of choice for a radiochemical assay of phospholipase C action on microsomal fractions.

Comparison of several methods for measuring phosphatidylcholine hydrolysis

Fig. 1(a) shows a close coincidence between two methods for measuring hydrolysis of phosphatidylcholine when microsomal fractions, labelled *in vivo* with radioactive choline, were treated with *Cl. perfringens* phospholipase C. In one case hydrolysis was measured radiochemically by the glass-fibre-disc method, whereas in the other case it was assayed chemically by determining P_i in wet-ashed residual phosphatidylcholine, which was isolated chromatographically. For times shorter than 15 min, there is a suggestion of slightly more rapid initial hydrolysis of the subset of phosphatidylcholines that are selectively labelled *in vivo* by radioactive choline, namely the 1-palmitoyl-2-oleoyl and the 1-palmitoyl-2-linoleoyl species (van den Bosch, 1974). However, when the deviations (which averaged 5.5%) in the determination of chromatographically separated phosphatidylcholine are taken into account, the two measures of hydrolysis overlap. In further experiments, using the more precise radiochemical phospholipase C assay on microsomal fractions double-labelled *in vivo* with [Me - 3 H]choline and [Me - 14 C]methionine, the two different subsets of selectively radioactively labelled phosphatidylcholines were hydrolysed at virtually identical rates by either *Cl. perfringens* or *B. cereus* phospholipases (Figs. 1b and 1d). [Me - 14 C]-Methionine selectively labels the hexaenoic species and possibly also the 1-stearoyl-2-arachidonoyl species of phosphatidylcholine (van den Bosch, 1974). Therefore, with labelling *in vivo*, it seems that selectivity of phosphatidylcholine hydrolysis is not so extensive as to interfere seriously with the radiochemical assay of phospholipase C action on microsomal fractions, or on ultrasonicated microsomal phosphatides (Fig. 1d).

When microsomal fractions are labelled *in vitro*

Fig. 1. Hydrolysis of different phosphatidylcholine species by phospholipase C

(a) Four samples of a [$Me-^{14}C$]choline-labelled microsomal fraction were treated at $5^{\circ}C$ with *Cl. perfringens* phospholipase C ($66 \mu g/mg$ of microsomal protein) and phosphatidylcholine hydrolysis was determined in timed samples both radiochemically (glass-fibre-disc method) and by the disappearance of P_i from phosphatidylcholine, extracted and separated by t.l.c. (Skipski *et al.*, 1964). Average deviations for P_i are shown, but for simplicity the smaller radioactivity deviations (mean $\pm 1.0\%$, range $0.6-1.6\%$), are omitted. $\bullet$, Phosphatidylcholine P_i ; $\circ$, radioactivity. (b) Microsomal fractions from rats double-labelled *in vivo* with $100 \mu Ci$ of [$Me-^3H$]choline and $4 \mu Ci$ of [$Me-^{14}C$]methionine were treated at $5^{\circ}C$ with *B. cereus* phospholipase C ($100 \mu g/mg$ of microsomal protein), and residual radioactive phospholipids were determined by counting radioactivity of extracted washed lipids from timed samples ($\bullet$, 3H ; $\circ$, ^{14}C). Exactly comparable results were obtained with *Cl. perfringens* phospholipase C (see also d). (c) Microsomal fractions from rats labelled *in vivo* with $50 \mu Ci$ of [$Me-^3H$]choline were double-labelled *in vitro* by incubating $1 \mu Ci$ of [$Me-^{14}C$]choline ($2.3 \mu g$) and $4 mM$ - Ca^{2+} (Bjerve, 1971). Then they were treated at $5^{\circ}C$ with *B. cereus* phospholipase C as in (b) in the presence of $10 mg$ of [^{12}C]choline/ml, and phospholipid hydrolysis was measured in timed samples by the disc method ($\bullet$, 3H ; $\circ$, ^{14}C ; $\blacksquare$, 3H in the absence of phospholipase; $\square$, ^{14}C in the absence of phospholipase). Exactly comparable results were obtained in three similar experiments with *Cl. perfringens* phospholipase C. (d) Microsomal fractions and washed microsomal lipids, the latter ultrasonically dispersed in $0.01 M$ -Tris/maleate buffer, pH 6.8 (Cater *et al.*, 1975), were double-labelled as in (b). Both were treated at $5^{\circ}C$ for 30 min with $0-200 \mu g$ of *Cl. perfringens* phospholipase C/ml, and residual radioactive phospholipids were determined by counting radioactivity of extracted washed lipids ($\bullet$, microsomal 3H ; $\circ$, microsomal ^{14}C ; $\square$, lipid 3H ; $\blacksquare$, lipid ^{14}C). Results for microsomal fractions are typical of three experiments.

by the Ca^{2+} -stimulated exchange of [$Me-^{14}C$]choline, this is selectively incorporated by the hexaenoic phosphatidylcholine species (Bjerve, 1971), but some hydrolysis by the microsomal phospholipase A also occurs (Bjerve *et al.*, 1974). Its partial conversion into a lyso form may partly explain why the [^{14}C]phosphatidylcholine subsequently appeared to be more slowly hydrolysed by phospholipase C, compared with [3H]phosphatidylcholine in the same microsomal fractions, which was labelled *in vivo* with [$Me-^3H$]choline

(Fig. 1c). However, controls, from which phospholipase C were omitted, suggest that an artifact may also contribute to the apparent slower hydrolysis of [^{14}C]phosphatidylcholine. These controls always showed a minor continued incorporation of ^{14}C (about 0.2% of that present), during the period of phospholipase C treatment, despite the presence of a 4350-fold excess of [^{12}C]choline (Fig. 1c). Microsomal binding of a minor radioactive impurity in the [^{14}C]choline might account for this. Finally, it should be

Fig. 2. Effect of pH on the activity and stability of phospholipase C

(a) Microsomal fractions labelled with $[Me-^{14}C]$ choline were treated for 60 min at $5^{\circ}C$ with $20 \mu g$ of *B. cereus* phospholipase C/ml over the pH range 4–8 in Na^{+} -free Buffer W, and the pH 9.0 point was obtained in 0.020 M-Tris buffer (Na^{+} -free). Phosphatidylcholine hydrolysis was measured by the disc method. Similar results were obtained in two experiments. (b) As (a) but $100 \mu g$ of *B. cereus* phospholipase C/ml used for 15 min at $5^{\circ}C$ in Buffer X. (c) Phosphatidylcholine hydrolysis by $20 \mu g$ of *B. cereus* phospholipase C/ml for 15 min at $37^{\circ}C$ in Na^{+} -free Buffer W was measured as in (a). The substrate was phospholipids, precipitated with acetone/ $MgCl_2$ from microsomal lipids labelled *in vivo* with $[Me-^3H]$ choline (Berry *et al.*, 1975) and ultrasonically dispersed in water. Similar results were obtained in three experiments and also at $5^{\circ}C$ and when the *Cl. perfringens* enzyme was used. (d) As in (a) and (b), but *Cl. perfringens* phospholipase C ($500 \mu g/ml$) was used for 15 min at $5^{\circ}C$ in Buffer X. Virtually identical results were obtained with both *Cl. perfringens* and *B. cereus* phospholipases in Buffer Y (results not shown). (e) Microsomal fractions from rats double-labelled *in vivo* with $100 \mu Ci$ of $[Me-^3H]$ choline and $10 \mu Ci$ of $[1,2-^{14}C]$ ethanolamine were treated with *B. cereus* phospholipase C in the Na^{+} -free Buffer W as in (a). Similar results were obtained in three experiments: 3H ; $\blacksquare$, ^{14}C ; $\bullet$. (f) *B. cereus* phospholipase C ($80 \mu g/ml$) was preincubated for 15 min at $37^{\circ}C$ with 0.001 M- Ca^{2+} in Buffer W at pH 4, 6 or 8 in the presence ($\blacksquare$) or absence ($\bullet$) of 3 mg of unlabelled microsomal fraction/ml. Microsomal fractions labelled *in vivo* with $[Me-^3H]$ choline were then added, and phosphatidylcholine hydrolysis was measured at $5^{\circ}C$ as in (a). Similar results were obtained in four experiments and also with *Cl. perfringens* phospholipase C.

noted that Graf & Stein (1966) detected slower hydrolysis of arachidonyl and eicosatrienoyl phosphatidylcholine species, compared with species containing oleate or linoleate, when ether solutions or aqueous dispersions of purified rat liver phosphatidylcholine were treated with *Cl. perfringens* phospholipase C.

Dependence of hydrolysis on pH

There was some inexplicable variability in the pH-dependence of phosphatidylcholine hydrolysis by the *B. cereus* and *Cl. perfringens* enzymes, with the activity at pH 4 and at pH 8–10 showing the greatest variations (Fig. 2). However, measurements in Na⁺-free (Figs. 2a, 2c and 2e) and Na⁺-containing buffers (Fig. 2b) suggest that inhibition by Na⁺ of the *B. cereus* enzyme did not markedly distort its pH-activity profile. Further, neither enzyme seemed noticeably unstable from pH 4 to 8 in the presence of microsomal fractions (Fig. 2f).

Though flat, the pH optima were found at a more acid pH than is generally reported (reviewed by Brockerhoff & Jensen, 1974), when either phospholipase was acting on phosphatidylcholine in microsomal fractions (Figs. 2a, 2b, 2d and 2e) or in lipid dispersions (Fig. 2c) or when the *Cl. perfringens* enzyme was hydrolysing microsomal phosphatidylethanolamine (Fig. 2e). Otnaess *et al.* (1972) also reported that one of their two pH optima for the *B. cereus* enzyme was in the acid region at pH 6.5. Figs. 2(a)–2(e) also clearly show the broad pH range over which these phospholipases exhibit substantial activity. This was also evident in the early studies of Macfarlane & Knight (1941) and Chu (1949), though modern reviews scarcely ever mention the relative insensitivity of these enzymes to pH, and it has apparently been assumed that shifting the pH from 8.0 to 5.5 or 5.75 will stop the action of *Cl. perfringens* phospholipase C (Zakim, 1970). An erroneous assumption is also frequently made that C-type phospholipases require exogenously added bivalent cations to hydrolyse membrane-bound phospholipids, though this has been clearly shown not to be the case: indeed, the *B. cereus* enzyme is active for some time, even in the presence of EDTA or EGTA [ethanedioxybis(ethylamine)tetra-acetate] [see Cater *et al.* (1975) for references]. Nevertheless, it is clear for the *Cl. perfringens* enzyme (Klein *et al.*, 1975), and probable for both phospholipases, that bivalent cations must be added to enable them to hydrolyse purified phospholipid, which is not membrane-bound.

We maintain that studies using C-type phospholipases, which may be contaminated by many other enzymes (Möllby *et al.*, 1973), can only be safely interpreted if phospholipase action on phospholipids is actually assessed. The availability of this simple and validated radiochemical assay, which can be made

extremely sensitive (Hallinan *et al.*, 1969) and can be adapted to follow the hydrolysis of several phospholipid classes either singly or simultaneously (Cater *et al.*, 1975), may encourage more regular monitoring of phospholipase C action on phospholipids, when these enzymes are used as tools to study membranes.

Aspects of this work were supported by the Science Research Council and the Wellcome Trust. B. R. C. was a Junior Research Fellow of the Royal Free Hospital School of Medicine.

References

- Akao, T. & Omura, T. (1972) *J. Biochem. (Tokyo)* **72**, 1245–1256
- Berry, C., Stellan, A. & Hallinan, T. (1975) *Biochim. Biophys. Acta* **403**, 335–344
- Bjerve, K. S. (1971) *FEBS Lett.* **17**, 14–16
- Bjerve, K. S., Daas, L. N. W. & Bremer, J. (1974) *Anal. Biochem.* **58**, 238–245
- Bjornstad, P. (1966) *Biochim. Biophys. Acta* **116**, 500–510
- Brockerhoff, H. & Jensen, R. G. (1974) *Lipolytic Enzymes*, pp. 275–276, Academic Press, New York
- Cater, B. R., Trivedi, P. & Hallinan, T. (1975) *Biochem. J.* **148**, 279–294
- Chu, H. P. (1949) *J. Gen. Microbiol.* **3**, 255–273
- Cope, G. H. & Williams, M. A. (1969) *J. Microsc. (Oxford)* **90**, 31–46
- Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957) *J. Biol. Chem.* **226**, 497–509
- Graf, E. & Stein, Y. (1966) *Biochim. Biophys. Acta* **116**, 166–169
- Grossman, S., Oestreicher, G. & Singer, T. P. (1974) *Methods Biochem. Anal.* **22**, 177–204
- Hallinan, T. (1974) *Biochem. Soc. Trans.* **2**, 817–821
- Hallinan, T., Nagley, P., Murty, C. N., Bennett, J. & Grant, J. H. (1969) *Biochim. Biophys. Acta* **173**, 554–563
- Klein, R., Miller, N., Kemp, P. & Laser, H. (1975) *Chem. Phys. Lipids* **15**, 15–26
- Lumper, V. L., Zubrzycki, Z. & Staudinger, H. (1969) *Hoppe-Seyler's Z. Physiol. Chem.* **350**, 163–172
- Macfarlane, M. G. & Knight, B. C. J. G. (1941) *Biochem. J.* **35**, 884–902
- Möllby, R., Nord, C. E. & Wadström, I. (1973) *Toxicon* **11**, 139–147
- Möllby, R., Thelestam, M. & Wadström, I. (1974) *J. Membr. Biol.* **16**, 313–330
- Nagley, P. & Hallinan, T. (1968) *Biochim. Biophys. Acta* **163**, 218–225
- Otnaess, A. B., Prydz, H., Bjorklid, E. & Berre, A. (1972) *Eur. J. Biochem.* **27**, 238–243
- Skipski, U. P., Peterson, R. F. & Barclay, M. (1964) *Biochem. J.* **90**, 374–378
- Stein, O. & Stein, Y. (1969) *J. Cell Biol.* **40**, 461–483
- van den Bosch, H. (1974) *Annu. Rev. Biochem.* **43**, 243–277
- White, D. A. (1973) in *Form and Function of Phospholipids* (Ansell, G. B., Hawthorne, J. N. & Dawson, R. M. C., eds.), pp. 441–482, Elsevier, Amsterdam
- Young, D. L., Powell, G. & McMillan, W. O. (1971) *J. Lipid Res.* **12**, 1–9
- Zakim, D. (1970) *J. Biol. Chem.* **245**, 4953–4961