A rapid and sensitive radiochemical assay for phosphatidic acid phosphohydrolase activity

Thomas J. Flynn,1 Diwakar S. Deshmukh,2 and Ronald A. Pieringer

Department of Biochemistry, Temple University School of Medicine, Philadelphia, PA 19140

Summary A technique is described for the radiochemical assay of phosphatidic acid phosphohydrolase activity in rat brain. Radiochemically pure $^{32}$P-labeled phosphatidic acid of known specific radioactivity and structure, which was biosynthesized in vitro by the diacylglycerol kinase of E. coli, was used as the substrate. As little as 5 μg of microsomal or mitochondrial protein can be used for the assay, and product formation in the picomole range can be determined accurately. This procedure should be useful in situations where only limited amounts of tissue are available.

Supplementary key words diacylglycerol kinase of Escherichia coli • rat • brain

Assay techniques for phosphatidic acid phosphohydrolase activity in brain tissue have been described previously (1–6). Most of these assays have been based on the procedure originally described by Smith, Weiss, and Kennedy (1), who prepared phosphatidic acid by treating phosphatidylincholine with phospholipase D. Release of phosphate from this substrate was quantitated by a colorimetric procedure. Some authors (4, 5) have indicated that phosphatidic acids prepared from phosphatidylcholine of different natural sources, as well as chemically prepared phosphatidic acids of known fatty acid composition, will serve as substrates for the phosphohydrolase reaction. However, there were observed differences in enzyme activity with the various phosphatidic acid preparations.

Phosphatidic acid phosphohydrolase activity has also been measured by a radiochemical technique (6). First, brain preparations were incubated with $^{32}$P-phosphate in order to label phospholipids. After addition of 2,4-dinitrophenol, which halted further incorporation of label, the incubation was allowed to proceed. Phosphohydrolase activity was then determined by extracting the lipids from the incubation mixture, separating them by thin-layer chromatography, and quantitating the decrease in radioactivity in the spot corresponding to phosphatidic acid relative to the radioactivity in the phosphatidic acid spot in the control sample.

The former assay techniques are limited by the sensitivity of the chemical determination of inorganic phosphate. The latter technique gains the added sensitivity that is obtained by using radioisotopes, but is somewhat awkward because of the necessity of a prolonged incubation period (i.e., for both incorporation and release of label) and the required purification of the unreacted substrate. Also, there is little or no control over substrate and enzyme concentrations used in the assay.

A procedure has been described for the biosynthetic preparation of radiochemically pure $^{32}$P-phosphatidic acid using an E. coli particulate enzyme (7). The enzyme (ATP: 1,2-diacyl-sn-glycerol-3-phosphotransferase) will accept a variety of diacylglycerols as substrates, making it possible to generate a phosphatidic acid of known fatty acid composition. In addition, the enzyme is stereospecific and produces only the naturally occurring 1,2-diacyl-sn-glycerol-3-phosphate (8). We have devised an assay technique for phosphatidic acid phosphohydrolase using biosynthetically prepared $^{32}$P-phosphatidic acid as substrate and rat brain as a source of enzyme. This new technique is rapid, straightforward, and highly sensitive.

Materials and Methods

Preparation of enzymes. Weanling Sprague-Dawley rats obtained from Charles River Breeding Laboratory, Wilmington, MA, were killed by decapitation. The whole brain was quickly removed, weighed, and homogenized in cold 0.32 M sucrose in a Potter-Elvehjem homogenizer. The final brain homogenate was 10% in sucrose. The homogenate was fractionated according to the procedure of DeRobertis et al. (9). The fractions collected were: crude nuclear fraction sedimenting at 900 g for 10 min; crude mitochondrial fraction sedimenting at 11,500 g for 20 min; microsomal fraction sedimenting at 100,000 g for 30 min; and post-100,000 g supernatant. The particulate fractions were lyophilized and stored at −20°C with a desiccant. The soluble fraction was frozen and stored at −20°C. Protein in each fraction was determined by the method of Lowry et al. (10) using crystallized bovine serum albumin (Sigma Chemical Co., St. Louis, MO) as a standard.

1 This work is part of a thesis submitted by T.J.F. in partial fulfillment of the requirements for the degree of Doctor of Philosophy. Present address, Department of Biology, Temple University, Philadelphia, PA 19122.

2 Present address, N.Y. Institute for Research in Mental Retardation, Staten Island, NY 10314.
TABLE 1. Effect of Triton X-100 on the release of inorganic phosphate from [32P]phosphatidic acid

<table>
<thead>
<tr>
<th>Triton X-100</th>
<th>Pi Released</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td>pmoI/hr</td>
</tr>
<tr>
<td>0</td>
<td>45</td>
</tr>
<tr>
<td>0.2</td>
<td>555</td>
</tr>
<tr>
<td>0.4</td>
<td>466</td>
</tr>
<tr>
<td>1.0</td>
<td>495</td>
</tr>
<tr>
<td>2.0</td>
<td>564</td>
</tr>
</tbody>
</table>

All assay tubes contained 8 μM [32P]phosphatidic acid, 0.1 M Tris-HCl buffer, pH 7.4, 5.0 μg of protein, and the indicated amount of Triton X-100, all in a total volume of 0.1 ml. Incubation was for 15 min at 37°C.

Preparation of [32P]phosphatidic acid. [32P]-Labeled phosphatidic acid was prepared enzymatically by the method of Pieringer and Kunnes (7). The incubation mixture consisted of the following: 0.5 mM 1,2-dipalmitoyl-sn-glycerol (Sigma), 0.5% Cutscum (Fisher Scientific Co., Pittsburgh, PA), v/v, 0.04 M sodium phosphate buffer, pH 7.0, 0.45 mg of a 30,000 g particulate suspension of E. coli (treated by placing a tube containing 1 ml of the suspension in a boiling water bath for 5 min to destroy phosphatidylcholine activity), 0.3 mM [γ-32P]ATP (New England Nuclear, Boston, MA), sp act adjusted with nonradioactive ATP to about 50 μCi/μmol, and 0.05 M MgSO4, all in a total volume of 0.49 ml. Incubation was performed for 45 min at 37°C. The reaction was stopped by the addition of 3 ml of 5% trichloroacetic acid. The precipitate was pelleted by centrifugation at 4,000 g for 10 min, and the pellet was washed twice with 5% trichloroacetic acid. The labeled phosphatidic acid was extracted from the pellet with 3 ml of chloroform–methanol 2:1. The extract was washed with 0.2 M sodium phosphate buffer, pH 6.0, in order to convert the free phosphatidic acid to a more stable sodium salt. The product was shown to be radiochemically pure by electrophoresis with authentic glycerol-3-phosphate after deacylation. The sp act of the phosphatidic acid can be calculated directly from the sp act of the [32P]ATP because endogenous phosphatidic acid does not accumulate in E. coli (11) and therefore does not alter the concentration of the phosphatidic acid generated in vitro. The decay of the [32P] label and the sp act of the phosphatidic acid was followed by determining the decline in the count rate of a reference planchette that had a known amount of [32P] on the day the [32P]phosphatidic acid was prepared. [32P] was counted with a gas-flow Geiger counter (Searle Analytic, Inc., Des Plaines, IL).

Enzymatic hydrolysis of [32P]phosphatidic acid. Phosphatidic acid phosphohydrolase activity was assayed in the following way. Two to three nmol of [32P]phosphatidic acid was added in chloroform solution to the assay tubes and the chloroform was removed with a stream of nitrogen. A 50-μl portion of 0.1 M Tris-HCl buffer, pH 7.4, containing 2% Triton X-100 was added, and the contents of the tubes were mixed on a Vortex mixer in order to disperse the substrate. The reaction was started by the addition of enzyme suspension containing about 5 μg of protein dispersed in 50 μl of 0.1 M Tris-HCl buffer, pH 7.4. Blank tubes received 50 μl of buffer only. Incubation was for 15 min at 37°C, and the reaction was stopped by addition of 3 ml of chloroform–methanol 2:1. One ml of water was added, and the tube contents were mixed and centrifuged. An aliquot of the aqueous layer was removed for counting. All radioactivity in the aqueous layer electrophoresed with inorganic phosphate. Enzyme activity was determined from the known sp act of the [32P]phosphatidic acid.

Results and discussion

In agreement with previous findings (1-5), over 90% of the total phosphatidic acid phosphohydrolase activity could be recovered in the crude mitochondrial and microsomal fractions of brain. Therefore, all further studies were carried out on these two fractions. Preliminary studies indicated anomalous behavior of the reaction at high substrate concentrations. Be-

![Fig. 1. Time course of the phosphatidic acid phosphohydrolase reaction, determined for both mitochondrial (■——■) and microsomal (○——○) enzymes. Each assay tube contained 25 μM [32P]phosphatidic acid, 0.05 M Tris-HCl buffer, pH 7.4, 1% Triton X-100, and about 5 μg of either crude mitochondrial or crude microsomal protein in a final volume of 0.1 ml. Incubations were carried out for the indicated times.](image-url)
cause of the detergent-like properties of phosphatidic acid, the reaction was examined in the presence of the nonionic detergent Triton X-100. As seen in Table 1, the reaction rate was increased about 10-fold by Triton X-100 at a concentration of 0.2%. Further increases in Triton X-100 concentration did not cause any additional stimulation. This observed stimulation by Triton X-100 is in contradiction to previous reports (1, 3, 5) which stated that detergents markedly inhibited phosphatidic acid phosphohydrolase activity.

As shown in Fig. 1, the reaction was linear for up to 15 min. The effect of enzyme protein at concentrations between 0.01 and 0.1 mg/ml is shown in Fig. 2. Except for a slight lag at the lowest concentrations of protein, the reaction rate appears to be linear for protein concentrations up to 0.075 mg/ml. The effect of substrate concentration on the amount of inorganic phosphorus liberated at a constant protein concentration and constant incubation time is shown as a Lineweaver-Burk plot in Fig. 3. The apparent $K_m$ values are 5 $\mu$M for the microsomal enzyme and 11 $\mu$M for the mitochondrial enzyme.

Prolonged storage of the enzyme preparations did not have any apparently adverse effects on enzyme activity. Lyophilized preparations stored at $-20^\circ$C for periods up to one year retained enzyme activity comparable to fresh preparations.

Previously published assays for phosphatidic acid phosphohydrolase (1–5) have utilized cold trichloroacetic acid (TCA) for terminating the reaction. Since these assays utilized a chemical test specific for inorganic phosphate, quantitative precipitation of unreacted phosphatidic acid was not essential. In the present assay the enzyme activity is determined from water-soluble radioactivity. Therefore, any phosphatidic acid not sedimented with the TCA-precipitable material would be counted along with the released phosphate and result in artifically high enzyme activities. Preliminary examination of the TCA-soluble material by paper electrophoresis indicated that a considerable amount of radioactivity due to unreacted phosphatidic acid had not sedimented with the precipitate. Therefore, the reaction was terminated by the addition of chloroform–methanol 2:1, and the products were partitioned by the addition of water. All radioactivity in the aqueous layer was shown on paper electrophoresis to be due to inorganic phosphate, while all radioactivity from unreacted phosphatidic acid remained in the chloroform layer.

Although assay techniques for phosphatidic acid phosphohydrolase activity in brain have been published (1–6), the technique described in this paper offers several advantages. First, the substrate can be tailor-made for the enzyme system being studied. Dipalmitoylglycerol was chosen as the diacylglycerol donor in the phosphatidic acid synthesis because palmitic acid is one of the major fatty acids in brain lipids (12). Other diacylglycerols would serve equally well in the synthesis of phosphatidic acid by the E. coli enzyme. Second, the relative ease in carrying out this assay makes it very attractive. In most tissues, including brain (13), the endogenous levels of phosphatidic acid are low and there is essentially
no dilution of the isotope. Because the sp act of the substrate is known, enzyme activity can be determined directly by counting the radioactivity in water-soluble products. However, a correction may be necessary if tissues with significant amounts of endogenous phosphatidic acid are used. Finally, this assay is highly sensitive. Enzyme activity was determined reliably using only 2 μg of protein from relatively crude enzyme preparations. The use of \(^{32}\text{P}\) also permitted the determination of the reaction product in pmol quantities, a level of sensitivity not possible with chemical assays for inorganic phosphate. This assay should, therefore, prove to be useful as a microtechnique in cases where either only small amounts of tissue are available, or enzyme activity is very low.

This investigation was supported by United States Public Health Service Research Grants AI-05730 and NS-10221, and by a Research Career Development Award (to R.A.P.) 1K4NS12776 from the National Institute of Neurological and Communicable Disorders and Stroke.

\textit{Manuscript received 1 December 1976 and accepted 26 May 1977.}

\textbf{REFERENCES}


