A Phosphodiesterase in Rat Kidney Cortex that Hydrolyses Glycerylphosphorylinositol

By R. M. C. DAWSON and N. HEMINGTON

Department of Biochemistry, Agricultural Research Council
Institute of Animal Physiology, Babraham, Cambridge CB2 4AT, U.K.

(Received 23 September 1976)

1. A phosphodiesterase, active at an alkaline pH, is present in the outer cortex of rat kidney and hydrolyses glycerylphosphorylinositol into glycerol and phosphorylinositol. Some inositol cyclic phosphate can also be formed indicating that the enzyme can act as a cyclizing phosphotransferase. 2. The enzyme is stimulated by Ca^{2+} (2–3 mM) whereas Mg^{2+} is inhibitory. 3. The activity is markedly stimulated by low concentrations of thiol reagents (1–2 mM) such as cysteine or dithiothreitol. 4. The properties of the enzyme have been compared with glycerylphosphinicocholine diesterase (EC 3.1.4.2), which is also present in the isolated enzyme complex, and it is concluded that the enzymes have separate identities.

It has become generally accepted that the catabolism of phosphatidylinositol occurs in tissues through a Ca^{2+} -activated phospholipase C type of activity which hydrolyses the phospholipid into diglyceride and inositol monophosphate (Dawson, 1959; Kemp *et al.*, 1961). More recently it has been shown that one of the primary products of the reaction is inositol 1:2-cyclic phosphate (Dawson *et al.*, 1971; Lapetina & Michell, 1973) and that a highly specific phosphodiesterase occurs in tissues which opens the ring to form inositol 1-phosphate (Dawson & Clarke, 1972).

However, there have been recent indications that in certain tissues most of the water-soluble combined inositol is in the form of glycerylphosphorylinositol (Seamark *et al.*, 1968; Koch & Diringer, 1974), and from time to time there have been reports that this substance can be formed from phosphatidylinositol by subcellular fractions from liver (Kemp *et al.*, 1961), intestinal mucosa (Atherton & Hawthorne, 1968) and brain (Quinn, 1973), and by seminal plasma (Scott & Dawson, 1968). We have identified a phospholipase in rat liver lysosomes that can completely deacylate phosphatidylinositol in the absence of divalent metal ions yielding glycerylphosphorylinositol (R. M. C. Dawson & R. Irvine, unpublished work).

The subsequent catabolism of glycerylphosphorylinositol in mammalian tissues has not been ascertained although it has been reported that glycerol phosphate was not split off from this phosphodiester by glycerylphosphinicocholine diesterase preparations (Baldwin & Cornatzer, 1968). An enzyme hydrolysing glycerylphosphorylinositol solely to glycerol phosphate and free inositol has, however,

been described in insect larvae (Hildenbrandt & Bieber, 1972). In the present paper we describe an enzyme present in rat kidney which degrades glycerylphosphorylinositol into free glycerol and either inositol monophosphate or inositol cyclic phosphate.

Experimental

Preparation of *sn*-glycero-3-phosphoryl-1-inositol

Crude soya phosphatidylinositol (85–90% pure, 15 mg of P) was dissolved in 4 ml of chloroform and 16 ml of 0.125 M-LiOH, H_2O in methanol was added. The mixture was left for 15 min at room temperature (18°C) to allow alkali-catalysed deacylation to occur; 60 ml of water, 40 ml of ethanol and 80 ml of chloroform were added. The mixture was shaken well and the upper phase separated by centrifuging (1000 g for 10 min) and then passed through an ion-exchange column (2 cm diam. $\times$ 15 cm) of Amberlite IRC 50 (H^+ form; BDH Chemicals, Poole, Dorset, U.K.) to remove the LiOH. The upper phase was washed through with 50% (v/v) ethanol, and the effluent and washings were adjusted to pH 7–8 with 2 M- NH_3 and concentrated to a low volume below 55°C *in vacuo*. The material was spotted as a preparative strip on paper chromatograms (Whatman no. 1, washed, loaded with 120 μg of P/cm) and developed unidimensionally in ethanol/12 M- NH_3 (3:2, v/v). The glycerylphosphorylinositol strip ($R_F^1 = 3.6$) was located by spraying marker lanes on either side to detect P and the material was eluted chromatographically with water. It was homogeneous when examined by paper ionophoresis or chromatography (Dawson & Clarke, 1972).

Other materials

Inositol 1:2-cyclic phosphate and inositol 1-phosphate were prepared as described (Dawson & Clarke, 1972); *sn*-glycero-3-phosphorylcholine was prepared from phosphatidylcholine (Dawson & Clarke, 1973).

Enzyme preparation

The enzyme used was a phosphodiesterase complex prepared from rat kidney microsomal fractions. The initial purification followed exactly the procedure described by Baldwin & Cornatzer (1968), involving solubilization of the membranes with sodium deoxycholate and $(\text{NH}_4)_2\text{SO}_4$ fractionation. The final product was then passed down a column (1.3 cm diam. $\times$ 63 cm long) of Sephadex G-200 equilibrated with 0.02 M-Tris/HCl buffer, pH 8.6. The activity is present in the initial high-molecular-weight peak completely excluded from the column: subsequent protein peaks were inactive.

Incubation. In general, the incubation medium contained 1 μmol of glycylphosphorylinositol, 0.01 M-Tris/HCl buffer, pH 8.6, 5 mM- CaCl_2 , 2.5 mM-cysteine and 0.1 mg of enzyme protein in a total volume of 0.85 ml, but variations occurred in individual experiments as stated in the legends. The mixtures were incubated for 60 min at 37°C and the reaction was terminated by adding 1 ml of 10% (w/v) trichloroacetic acid at 0°C.

Determination of enzymic products. After centrifuging the fixed incubation mixture, the supernatant was washed four times by shaking on each occasion with twice its volume of diethyl ether to remove the trichloroacetic acid. It was adjusted to pH 7–8 with aq. NH_3 , concentrated to a small volume as above and spotted on to paper (Whatman no. 1). Ionophoresis was carried out in a pyridine/acetic acid buffer, pH 3.6, for 2 h at 50 V/cm (Dawson & Clarke, 1972). The inositol monophosphate produced by the enzyme, together with any inositol cyclic phosphate and P_i (if present), were located and estimated by methods described by Dawson & Clarke (1972). The separation between inositol monophosphate and glycylphosphorylinositol was adequate provided the buffer concentration in the incubation medium was kept low. Control incubations showed virtually no non-enzymic decomposition of the substrate.

In certain experiments designed to identify the non-phosphorus-containing product of the reaction, the reaction was terminated by shaking the incubation mixture with 5 ml of chloroform/methanol (2:1, v/v). The separated upper phase was concentrated to a small volume and ionophoresed as above. For the detection of glycerol, the dried ionophoretogram was developed chromatographically in propan-1-ol/ethyl acetate/water (7:1:2, by vol.) to separate the uncharged compounds that had remained near the origin after ionophoresis. Polyols on these sheets

were detected by the reagents of Trevelyan *et al.* (1950), glycerol giving a characteristic jet-black coloration.

Reproducibility

All experiments described in the text were repeated at least three times with minor variations in technique and incubation conditions, and the results were similar in all essential details.

Results

Identification of products of enzymic reaction

The possible P-containing products of the enzymic decomposition of glycylphosphorylinositol could be readily separated and characterized by paper ionophoresis at pH 3.6 (inositol 1-phosphate, m_{Rf} 0.69; inositol 1:2-cyclic phosphate, m_{Rf} 0.76; *sn*-glycerol 3-phosphate, m_{Rf} 0.84), and the substrates mobility relative to P_i was 0.64. Usually the predominant P-containing product detectable in incubation mixtures was inositol monophosphate with traces of the cyclic derivative. When crude enzyme preparations were used, e.g. microsomal preparations, larger amounts of inositol cyclic phosphate occurred but inositol monophosphate was still the dominant product. No glycerol phosphate was ever detected, although traces of P_i were sometimes liberated when cysteine was omitted from the incubation medium.

Paper chromatography of the incubation products showed the production of free glycerol with the same mobility and colour response as glycerol markers. No free inositol was formed unless alkaline phosphatase activity had occurred. The intensity of the enzymically produced glycerol spot, compared visually with a graded series of glycerol marker concentrations, indicated that the glycerol produced was approximately stoichiometric to the inositol monophosphate formed.

General characteristics of the enzymic reaction

Under the incubation conditions stated the rate of reaction was proportional to enzyme concentration up to 200 μg of protein/ml of medium, and approximately linear with time up to 60 min. The K_m of the reaction was 2.2 mM. The pH optimum measured in a series of Tris/HCl and 2-amino-2-methylpropane-1,3-diol buffers was broad: pH 7.8–8.6.

Effect of thiol-containing reducing reagents on the reaction

The basic reaction in the presence of Ca^{2+} ions was markedly stimulated by the addition of thiol-containing reducing agents, e.g. cysteine, dithiothreitol or mercaptoethanol (Fig. 1). Cysteine was the most effective of those tried, and it also had the advantage that it is an effective inhibitor of alkaline phosphatase

Fig. 1. Stimulation of glycerylphosphorylinositol degradation by thiols

The incubation conditions were as described in the Experimental section except different concentrations of cysteine (○) or alternatively dithiothreitol (●) or mercaptoethanol (△) were added.

Fig. 2. Effect of bivalent ions and EDTA on the hydrolysis of glycerylphosphorylinositol

The incubation conditions were as described in the Experimental section except different concentrations of CaCl_2 (○) or alternatively MgCl_2 (●) or MnCl_2 (△) or EDTA (disodium salt) (▲) were added.

(Cooper & Hawthorne, 1975). Some enzyme preparations contained a little residual alkaline phosphatase, which resulted in the partial dephosphorylation of the inositol phosphate produced if cysteine was absent.

Activation by bivalent ions

Although some activity occurred in the absence of added bivalent ions, the addition of Ca^{2+} markedly

stimulated the reaction with maximum stimulation at approx. 2–3 mM- CaCl_2 (Fig. 2). Mn^{2+} produced a more limited stimulation at low concentrations and Mg^{2+} was inhibitory (Fig. 2). Zn^{2+} produced no activation; EDTA completely suppressed even the basic reaction seen in the absence of added bivalent ions (Fig. 2).

Location of enzyme

Rat kidney was separated into outer cortex, inner cortex and medullary fractions, and homogenates of each were prepared and tested for enzymic activity. Activity was almost entirely confined to the outer cortex of the kidney. Histochemical location of the enzyme in frozen sections of rat kidney by using *Escherichia coli* phosphomonoesterase and a Gomori-type technique (Clarke & Dawson, 1972) confirmed this localization in the outer cortex, and, in addition,

Fig. 3. Effect of bivalent ions on the hydrolysis of glycerylphosphorylcholine and inositol 1:2-cyclic phosphate by the enzyme complex

The incubation conditions were those described in the Experimental section except the substrate used was glycerylphosphorylcholine (a) or inositol 1:2-cyclic phosphate (b). Various concentrations of MgCl_2 (●) or CaCl_2 (○) were added. When inositol 1:2-cyclic phosphate was used as substrate the protein used was decreased to 40 µg. The hydrolysis products (glycerol phosphate or inositol monophosphate) were separated by ionophoresis and estimated as stated in the Experimental section.

indicated that the activity was concentrated in the brush borders of the proximal tubules. Evidence has also been obtained of glycerylphosphorylinositol hydrolysing activity in a rat liver microsomal preparation, and also in rat spleen homogenates.

Comparison of enzyme with other phosphodiesterases

The enzyme preparation presumably represents a complex of phosphodiesterases with alkaline pH optimum, since glycerylphosphorylcholine and inositol 1:2-cyclic phosphate could also be hydrolysed. Glycerylphosphorylcholine was exclusively converted into glycerol phosphate, and no evidence was obtained for the formation of phosphorylcholine as described by Abra & Quinn (1975) for the breakdown of this substrate by brain homogenates. Under the standard conditions of incubation used here, cysteine did not activate the hydrolysis of glycerolphosphorylcholine. Its breakdown was stimulated by the addition of Ca^{2+} but, unlike that of glycerylphosphorylinositol, also by Mg^{2+} (Fig. 3). This stimulation of glycerylphosphoricocholine diesterase by bivalent ions is in contrast with the results of Baldwin & Cornatzer (1968), but agrees with those of Dawson (1956) and Webster *et al.* (1957).

In addition, the hydrolysis of glycerylphosphorylinositol showed a small but consistent higher thermal instability at 60°C compared with glycerylphosphoricocholine diesterase when tested by the method of Baldwin & Cornatzer (1968). Glycerylphosphorylcholine in a sixfold excess of substrate compared with glycerylphosphorylinositol did not inhibit the hydrolysis of the latter substrate, and the same was true for the reverse situation, i.e. glycerylphosphorylinositol had no effect on the hydrolysis of glycerylphosphorylcholine. The enzyme preparation hydrolysed inositol cyclic phosphate to inositol monophosphate (Dawson & Clarke, 1972), but again, unlike glycerylphosphorylinositol hydrolysis, this hydrolysis was activated by Mg^{2+} ions.

Discussion

The large but soluble enzyme complex isolated from the kidney microsomal fraction undoubtedly has the ability to hydrolyse glycerylphosphorylinositol into inositol monophosphate and free glycerol. The failure of Baldwin & Cornatzer (1968) to detect hydrolysis of this substrate with a similar enzyme preparation is almost certainly due to their more limited analysis of the reaction products, which was confined to glycerol phosphate by using α -glycerol phosphate dehydrogenase. No schism of the molecule to liberate glycerol phosphate, as occurs with the phosphodiesterase action on glycerylphosphorylcholine, could be detected in our studies. The enzyme is clearly different from the hydrolase that degrades glycerylphosphorylinositol described by Hilden-

brandt & Bieber (1972) in insect larvae, since this liberates glycerol phosphate and free inositol exclusively. The site of enzymic attack is more equivalent to the hydrolysis of glycerylphosphorylcholine into glycerol and phosphorylcholine (EC 3.1.4.38), as described by Abra & Quinn (1975) in rat brain. However, our enzyme preparation hydrolysed glycerylphosphorylcholine to produce exclusively glycerol phosphate: no phosphorylcholine was formed.

In many of the enzyme incubation mixtures, trace and sometimes larger amounts of inositol cyclic phosphate could be detected, although inositol monophosphate was always the predominant product. Since the enzyme complex also contains inositol 1:2-cyclic phosphate 2-phosphohydrolase, which rapidly hydrolyses added inositol 1:2-cyclic phosphate to inositol monophosphate under the incubation conditions used, it is reasonable to assume that substantial amounts of inositol cyclic phosphate are formed in the primary reaction. Thus the hydrolytic enzyme can also act as an intramolecular cyclizing phosphotransferase at the time of schism, so that the phosphate rings to the 2-position on the inositol ring. This is equivalent to the Ca^{2+} -requiring phospholipase degrading phosphatidylinositol, which can produce inositol cyclic phosphate as a primary product (Dawson *et al.*, 1971).

Since the large enzyme complex isolated has the ability to break down other phosphodiesterases apart from glycerylphosphorylinositol, namely glycerylphosphorylcholine and inositol cyclic phosphate, the question arises as to whether the enzyme involved has a specific identity. Like glycerylphosphoricocholine diesterase and inositol 1:2-cyclic phosphate 2-phosphohydrolase (Dawson & Clarke, 1973), the ability to hydrolyse glycerylphosphorylinositol is rather specifically located in the outer cortical region of rat kidney and in the brush borders of the proximal tubules.

The present hydrolysis of glycerylphosphorylinositol is unique since whereas it is inhibited by the addition of Mg^{2+} , hydrolysis of the other phosphodiesterase substrates is markedly activated by Mg^{2+} addition. The hydrolysis of glycerylphosphorylcholine by the enzyme complex was also insensitive to cysteine addition and showed a somewhat higher thermal stability. In addition, no competitive inhibition of the hydrolysis of glycerylphosphorylinositol by glycerylphosphorylcholine or vice versa could be detected.

Although, in spite of much effort, we have been unable to achieve a significant separation from the enzyme complex of independent phosphodiesterases hydrolysing glycerylphosphorylinositol and glycerylphosphorylcholine, the above observations, together with the different sites of attack of the enzymes on the analogous molecules, would indicate a separate identity. We have succeeded in separating kidney glycerylphosphoricocholine diesterase and inositol 1:2-cyclic

phosphate 2-phosphohydrolase by fractionation of the complex (N. G. Clarke & R. M. C. Dawson, unpublished work), so it would appear that the brush-border membranes of the kidney proximal tubule contain a tightly adhering complex of different phosphodiesterases which serves some, as yet unknown, physiological function concerned with filtration or reabsorption.

There are indications that the present enzyme also occurs in other tissues apart from kidney, and consequently, if coupled with a lysosomal phospholipase deacylating phosphatidylinositol, this would represent a different method for the catabolism of this phospholipid in mammalian tissues, distinct from the diglyceride/inositol monophosphate-forming phospholipase. It remains an open question as to the quantitative importance of each pathway both in the basic turnover of phosphatidylinositol and the greatly increased turnover observed in many cells during stimulation (Michell, 1975). The preferential loss of ^{32}P compared with the ^3H -labelled glycerol moiety from phosphatidylinositol after pulse-labelling reported by some workers has been ascribed to recycling of the diglyceride moiety split off (Diringer, 1973), but other interpretations are possible. In addition, more recent studies with SV-40 virus-transformed mouse tissue cells have indicated that ^{32}P and $[^3\text{H}]$ glycerol are lost together from the bulk of the rapidly metabolized phosphatidylinositol pool (Koch & Diringer, 1974).

References

- Abra, R. M. & Quinn, P. J. (1975) *Biochim. Biophys. Acta* **380**, 436–441
- Atherton, R. S. & Hawthorne, J. N. (1968) *Eur. J. Biochem.* **4**, 68–75
- Baldwin, J. J. & Cornatzer, W. E. (1968) *Biochim. Biophys. Acta* **164**, 195–204
- Clarke, N. & Dawson, R. M. C. (1972) *Biochem. J.* **130**, 229–238
- Cooper, P. H. & Hawthorne, J. N. (1975) *Biochem. J.* **150**, 537–551
- Dawson, R. M. C. (1956) *Biochem. J.* **62**, 689–696
- Dawson, R. M. C. (1959) *Biochim. Biophys. Acta* **33**, 68–77
- Dawson, R. M. C. & Clarke, N. G. (1972) *Biochem. J.* **127**, 113–118
- Dawson, R. M. C. & Clarke, N. G. (1973) *Biochem. J.* **134**, 59–67
- Dawson, R. M. C., Freinkel, N., Jungalwala, F. B. & Clarke, N. (1971) *Biochem. J.* **122**, 605–607
- Diringer, H. (1973) *Hoppe-Seyler's Z. Physiol. Chem.* **354**, 577–582
- Hildenbrandt, G. R. & Bieber, L. L. (1972) *J. Lipid Res.* **13**, 348–355
- Kemp, P., Hübscher, G. & Hawthorne, J. N. (1961) *Biochem. J.* **79**, 193–200
- Koch, M. A. & Diringer, H. (1974) *Biochem. Biophys. Res. Commun.* **58**, 361–367
- Lapetina, E. G. & Michell, R. H. (1973) *Biochem. J.* **131**, 433–442
- Michell, R. H. (1975) *Biochim. Biophys. Acta* **415**, 81–147
- Quinn, P. J. (1973) *Biochem. J.* **133**, 273–281
- Scott, T. W. & Dawson, R. M. C. (1968) *Biochem. J.* **108**, 457–463
- Seamark, R. F., Tate, M. E. & Smeaton, T. C. (1968) *J. Biol. Chem.* **243**, 2424–2428
- Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1950) *Nature (London)* **166**, 444–445
- Webster, G. R., Marples, E. A. & Thompson, R. H. S. (1957) *Biochem. J.* **65**, 374–377