D-myoInositol 1:2-Cyclic Phosphate 2-Phosphohydrolase

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

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

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1. An enzyme in extracts of mammalian tissues catalyses the hydrolysis of D-myoinositol 1:2-cyclic phosphate (an intermediary in the enzymic degradation of phosphatidylinositol) to produce D-myoinositol 1-phosphate. 2. The enantiomorph of the substrate is not attacked. 3. The pH optimum is about 8.1–8.3 and the reaction is stimulated by Mg^{2+} ions. 4. Extracts from rat kidney cortex and medulla are very rich sources of the enzyme; brain, testis and small intestine contain intermediary activities, and other tissues contain very small amounts.

It has recently been shown that the enzyme in tissues which degrades phosphatidylinositol liberates D-inositol 1:2-cyclic phosphate as the main water-soluble product (Jungalwala *et al.*, 1971; Dawson *et al.*, 1971) rather than D-inositol 1-phosphate as had previously been assumed (Dawson, 1959; Kemp *et al.*, 1961; Thompson, 1967; Atherton & Hawthorne, 1968; Friedel *et al.*, 1969).

In the present paper we report that there is a phosphodiesterase in tissues which hydrolyses inositol 1:2-cyclic phosphate to inositol 1-phosphate. The enzyme is present in high concentrations in kidney cortex and is specific for the D isomer of inositol 1:2-cyclic phosphate.

Methods

Preparation of phosphatidylinositol

Phosphatidylinositol was prepared from pig liver. The lipids were extracted from 3 kg of tissue by the procedure of Bligh & Dyer (1959). The washed lipids were taken up in a minimum volume of chloroform and precipitated with 250 ml of ethanol at -10°C . Repetition of this ethanol precipitation largely removed the phosphatidylcholine (ethanol-soluble) from the ethanol-insoluble phospholipids, including phosphatidylinositol. The ethanol-insoluble lipids were applied to an alumina column (300 g of aluminium oxide 'Camag'; Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K.) in chloroform-methanol (9:1, v/v) and eluted successively with (a) 1 litre of chloroform-methanol-water (23:23:4, by vol.), (b) 1 litre of chloroform-methanol-water (43:43:14, by vol.), followed by (c) 4 litres of chloroform-ethanol-water (2:5:2, by vol.) and then (d) chloroform-ethanol-0.55 M- NH_3 (2:5:2, by vol.). Fractions were collected in a fraction collector and monitored by t.l.c. Eluate (b) did not contain phosphatidylinositol, as had been expected from the

report of Long & Owens (1966), but this was eluted eventually by solvent (c), with phosphatidylethanolamine preceding it in the same solvent. Solvent (d) eluted some more phosphatidylinositol together with cardiolipin and a little phosphatidylserine. The phosphatidylinositol (16.5 mg of P) eluted by solvent (c) was used directly whereas that (8 mg of P) eluted by solvent (d) was further purified by chromatography on a silicic acid column to remove cardiolipin (Long & Owens, 1966). ^{32}P -labelled phosphatidylinositol was prepared from *Saccharomyces cerevisiae* lipids, labelled, extracted and fractionated as described by Bangham & Dawson (1960) and Hauser & Dawson (1967). The lipids remaining on the alumina column were eluted with solvent (c) and the [^{32}P]phosphatidylinositol was prepared from the eluate by preparative t.l.c. (Scott & Dawson, 1968).

Preparation of inositol 1:2-cyclic phosphate

D-Isomer. A chloroform solution of phosphatidylinositol (1 mg of P) was evaporated to dryness and mixed with 4 ml of sodium acetate-acetic acid buffer (pH 5.6, 0.1 M) and 2.4 ml of 0.01 M- CaCl_2 . Sufficient purified phosphatidylinositol-degrading enzyme from ox liver (25–40% satd.-ammonium sulphate fraction; Kemp *et al.*, 1961) was added, to effect 80% breakdown of the substrate in 1 h incubation at 37°C in 10 ml total volume. To the final incubation medium was added 16 ml of methanol and 32 ml of chloroform. After shaking, the upper phase was collected and washed with theoretical lower phase (Folch *et al.*, 1957) containing 0.1 M- CaCl_2 . The upper phase was subjected to preparative paper ionophoresis [four strips 40 cm wide on Whatman no. 1 paper at pH 3.6 (pyridine-acetic acid buffer)] for 3 h at 40 V/cm. Marker strips were sprayed to locate the D-inositol 1:2-cyclic phosphate (m_R 0.76), which ran just in advance of the inositol 1-phosphate (m_R 0.70) also formed in the enzymic reaction (Dawson *et al.*, 1971).

Radioautography was used for location when [^{32}P]-phosphatidylinositol had been employed as substrate. The strips containing the cyclic compound were chromatographically eluted with water and the P contained in the eluate was determined (Bartlett, 1959). The cyclic compound did not decompose when the solution was deep-frozen at pH 8 (yield, approx. 50% of starting phospholipid P).

DL-Compound. The racemic compound was synthesized by the method of Pizer & Ballou (1959) from inositol 2-phosphate (Sigma Chemical Co., St. Louis, Mo., U.S.A.). The DL-myoinositol 1:2-cyclic phosphate was not crystallized but was purified from the reaction mixture by the ionophoretic procedure used for the D-isomer. Both the enzymically produced and the synthesized compounds showed identical behaviour on ionophoresis and paper chromatography (Table 1).

Phosphodiesterase activity

In a typical incubation 0.5 μmol of D-inositol 1:2-cyclic phosphate labelled with ^{32}P (10000–20000 c.p.m., and usually consisting of a mixture of the unlabelled and labelled preparations) was added to a medium containing 0.02 M-2-amino-2-methylpropane-1,3-diol-HCl buffer, pH 8.3, and 10 mM-MgCl₂, total volume 0.85 ml. The enzyme preparation was a water homogenate of the tissue (1 g/9 ml, 0°C, Potter homogenizer) centrifuged at 10000g for 20 min, and the supernatant dialysed against water overnight. After incubation at 37°C for 20–40 min, 1.4 ml of methanol followed by 2.8 ml of chloroform were added and the mixture was shaken and centrifuged. The upper aqueous layer was removed and the lower chloroform layer washed with 0.5 vol. of chloroform-methanol-water (3:45:47, by vol.). The aqueous

layer and washings were concentrated to low volume below 50°C and applied to a paper for ionophoresis. Ionophoresis was carried out in a buffer at pH 3.6 (pyridine-acetic acid) for 2 h at 40 V/cm under white spirit. Spots of P_i, inositol monophosphate and inositol cyclic phosphate were located by spraying to detect P (Dawson, 1959), the spots were cut out and digested and the ^{31}P and ^{32}P contained in them measured by the assay of Bartlett (1959) and by Čerenkov counting (Jungalwala *et al.*, 1971).

Results

Preliminary experiments with rat liver homogenates in water showed that the supernatant obtained by centrifuging at 10000g for 20 min contained enzymes that broke down D-inositol 1:2-cyclic phosphate, mainly into inositol monophosphate but with some P_i liberation. This suggested that there was an enzyme present in the extract that opened the phosphorus-containing ring present in inositol 1:2-cyclic phosphate and that the inositol monophosphate released was subsequently decomposed by phosphomonoesterase activity. Later experiments with pig kidney cortex showed that 60% of the activity in a similar supernatant fraction could be sedimented by centrifuging at 100000g_{av.} for 45 min and that the specific activity in the precipitate was nearly four times that in soluble protein. The enzyme responsible for hydrolysis of the cyclic phosphate ester is possibly therefore attached to membrane fragments.

Identification of the main hydrolysis product as myo-inositol 1-phosphate

Table 1 shows the paper-chromatographic and ionophoretic properties of various phosphate esters

Table 1. *Paper-chromatographic and ionophoretic properties of phosphate esters containing myoinositol*

Values represent the distance travelled by the substances in cm.

	Descending chromatography in ethanol–13.5 M-NH ₃ (3:2, v/v; 18 h)	Descending chromatography in propan-2-ol–6 M-NH ₃ (7:3, v/v; 40 h at 30°C)	Ionophoresis at pH 3.6 (pyridine–acetic acid buffer) (2.5 h at 40 V/cm)
DL-Inositol 1:2-cyclic phosphate	37.4	26.4	30.5
D-Inositol 1:2-cyclic phosphate	37.4	26.4	30.3
P _i	10.6	14.4	39.9
Inositol 1-phosphate*	15.0	14.3	27.9
Inositol 2-phosphate	19.3	18.2	28.5
Glycerol 2-phosphate	29.2	24.4	34.0
Glycerylphosphorylinositol†	38.2		25.5

* Prepared by the method of Brown *et al.* (1959).

† Prepared by deacylation of phosphatidylinositol (Dawson, 1954).

of inositol in comparison with P_i and β -glycero-phosphate. The ethanol- NH_3 solvent gave a good separation of inositol 1- and 2-phosphate, P_i and inositol 1:2-cyclic phosphate. The solvent used by Pizer & Ballou (1959) also separated inositol 1- and 2-phosphate but the very fast mobility relative to glycerol 2-phosphate for inositol 1:2-cyclic phosphate reported by these workers could not be confirmed. In experiments designed to determine the isomer of inositol phosphate produced by the enzyme, a brain supernatant fraction was used, since this readily converted inositol 1:2-cyclic phosphate into inositol monophosphate, which was then only slowly hydrolysed to inositol and P_i (Table 3). The supernatant

was allowed to decompose inositol 1:2-cyclic [^{32}P]-phosphate and the resulting products were subjected to two-dimensional separation on paper with ethanol- NH_3 solvents and ionophoresis (Table 1). Radioautography showed that the ^{32}P -labelled metabolic product was exclusively associated with an inositol 1-phosphate marker, no radioactivity being detected in the inositol 2-phosphate position.

pH optimum

With a pig kidney supernatant, breakdown of D-inositol 1:2-cyclic phosphate occurred most rapidly at pH 8.1-8.3 (Fig. 1). Similar results were obtained with a rat kidney extract. Rat liver supernatant produced inositol monophosphate most actively at pH 8.3 but there was also a minor peak in the pH profile at about pH 6.

Effect of bivalent metal ions

Although some activity occurred in the absence of added Mg^{2+} the latter produced a marked stimulation of the reaction (Table 2); under the present incubation conditions 10mM was usually sufficient to produce maximum stimulation. Ca^{2+} and Mn^{2+} (5mM) were inactive. The basal release in the absence of Mg^{2+} was almost entirely suppressed by the addition of 20mM-EDTA (sodium salt).

Activity towards substrate enantiomorphs

Experiments were made to test whether the hydrolysing enzyme was active towards both D- and L-forms of inositol 1:2-cyclic phosphate. Since the inositol 1-phosphate prepared from phosphatidyl-inositol has the D configuration (Ballou & Pizer, 1960) according to the present CBN rules for cyclitol nomenclature [*Biochem. J.* (1969) 112, 17] it has been assumed that the enzyme degrading

Fig. 1. Activity of inositol 1:2-cyclic phosphate-hydrolysing enzyme of pig kidney in various buffers

Experimental details are given in the text. Buffers (0.03M) were as follows: $\circ$, $\beta\beta$ -dimethylglutaric acid-NaOH; Δ , 2,4,6-trimethylpyridine-HCl; $\square$, 2-amino-2 methylpropane-1,3-diol-HCl.

Table 2. Effect of Mg^{2+} ions on the hydrolysis of D-inositol 1:2-cyclic phosphate

The supernatant fraction (liver $\equiv$ to 15mg wet wt.; kidney $\equiv$ 1.3mg wet wt.) was incubated with D- [^{32}P]inositol 1:2-cyclic phosphate (0.5 μ mol) and 0.025M-2-amino-2-methylpropane-1,3-diol-HCl buffer, pH 8.3, in a total volume of 0.5ml.

Enzyme and incubation time	Mg^{2+} (mM)	Substrate hydrolysed (μ g of P)
Rat liver supernatant dialysed: 60min incubation	0	1.3
	0.33	2.3
	1.3	3.2
	6.7	4.4
Rat kidney-cortex supernatant dialysed: 25 min incubation	0	0.4
	0.25	0.8
	1.0	1.0
	2.5	1.4
	5.0	2.2

Fig. 2. Comparison of the radioactivity and phosphorus remaining in a substrate consisting of D-inositol 1:2-cyclic [^{32}P]phosphate diluted with either D- or DL-inositol 1:2-cyclic phosphate (unlabelled)

The enzyme in a brain supernatant was allowed to attack the mixed substrate for various times and the radioactivity and phosphorus remaining in the substrate after reisolation from the incubation medium determined. ○, D-Inositol 1:2-cyclic [^{32}P]phosphate (0.55 μg of P) + D-inositol 1:2-cyclic phosphate (12.0 μg of P); ●, D-inositol 1:2-cyclic [^{32}P]phosphate (0.55 μg of P) + DL-inositol 1:2-cyclic phosphate (14 μg of P).

phosphatidylinositol would produce D-inositol 1:2-cyclic phosphate. Radioactive D-inositol 1:2-cyclic [^{32}P]phosphate was mixed with about 24 times its weight of unlabelled D- or DL-inositol 1:2-cyclic phosphate. When the mixture was attacked by the enzyme contained in a brain supernatant fraction there was the expected correlation of phosphorus and radioactivity disappearing from the substrate when the diluent was the D isomer (Fig. 2). However, when the diluent was DL-inositol 1:2-cyclic phosphate the radioactivity was released from the substrate at a much faster rate than the total phosphorus. By extrapolation to zero radioactivity remaining in the substrate (Fig. 2) 7.4 μg of P would remain as inositol 1:2-cyclic phosphate. This is approximately half of the phosphorus added in the diluent (14 μg of P in DL-inositol 1:2-cyclic phosphate) and indicates therefore that the enzyme cannot attack L-inositol 1:2-cyclic phosphate. Similar results were obtained with rat liver and spleen extracts.

Distribution of enzyme in extracts from various tissues

Table 3 shows the ability of extracts from various tissues of the rat to break down D-inositol 1:2-cyclic phosphate at pH 8.3. Although some variation was found between individual rats, by far the most active tissue extracts examined were kidney cortex and medulla, followed by brain, testis and small intestine, with heart and skeletal muscle and liver showing rather low activity. With small intestine supernatant the inositol monophosphate liberated was extensively broken down to P_i , presumably by the alkaline phosphatase contained in the enzyme preparation. No activity could be detected in rat blood. High activities were also obtained with ox and pig kidney extracts, and as with rat, the specific activity of the enzyme in the kidney-cortex supernatant was higher than that in the medulla. In ox brain the extract of white matter had almost double the specific activity of that obtained from grey matter.

Discussion

It has long been realized that phosphatidylinositol has a turnover in mammalian tissues which is considerably faster than that of most of the other membrane phospholipids and is similar to that shown by phosphatidic acid, an intermediate in their synthesis. Moreover a rather specific enhancement of phosphatidylinositol turnover has been observed to accompany increased functional activity in certain tissues, e.g. when the thyroid is stimulated by thyroid-stimulating hormone (Scott *et al.*, 1968), or an excised sympathetic ganglion is stimulated through its pre-ganglionic nerve (Larrabee & Leicht, 1965). It has been assumed that the catabolism of the phosphatidylinositol during this turnover was by a breakdown of the phospholipid into diglyceride and inositol monophosphate. However, the isolated soluble enzyme that degrades phosphatidylinositol produces, at its pH optimum (5.6), mainly inositol 1:2-cyclic phosphate rather than inositol 1-phosphate (Dawson *et al.*, 1971). It is, of course, not possible to deduce from this the extent to which the cyclic compound is formed in tissues *in vivo*. However, that such a formation occurs in the intact animal is made far more likely by the present finding in tissues of an enzyme that degrades the cyclic phosphate ester. The hydrolysis occurs specifically at the bond between the phosphate atom and the 2-position on the inositol ring, releasing inositol 1-phosphate. This type of specificity is not unknown among the phosphodiesterases, since it has been reported that those found in spleen, pancreas and brain specifically convert ribonucleoside 2':3'-cyclic phosphates into the corresponding ribonucleoside 2'-phosphates (Drummond *et al.*, 1962). However, that the breakdown is not carried out by a non-specific phospho-

Table 3. *Activity of the phosphodiesterase in extracts of various tissues*

Suitable samples of tissue supernatant were incubated (20 min, 37°C) with D-[³²P]inositol 1:2-cyclic phosphate (0.5 μmol), 0.17 M-2-amino-2-methylpropane-1,3-diol-HCl buffer, pH 8.3, and 6 mM-MgCl₂ in a total volume of 0.45 ml.

Species	Tissue	Activity (μg of P liberated/h)			
		(per 10 mg of tissue)			(per mg of protein)
		Inositol monophosphate	P _i	Total	Total
Rat	Brain	5.31	0.36	5.67	9.22
	Heart	0.96	0.06	1.02	2.90
	Liver	1.14	0.15	1.29	1.24
	Skeletal muscle	0.36	0.06	0.42	1.58
	Spleen	1.98	0.15	2.13	1.81
	Small intestine	0.24	1.50	1.74	4.98
	Testis	4.14	0.75	4.89	7.11
	Kidney medulla	43.2	4.2	47.4	78.0
	Kidney cortex	192.6	15.9	208.5	171.0
	Blood	0	0	0	0
Ox	Brain grey matter	1.64	0.14	1.78	5.02
	Brain white matter	1.96	0.11	2.07	9.21
	Cerebellum	1.91	0.12	2.03	7.51
	Spinal cord	1.16	0.15	1.31	5.20
Pig	Kidney cortex			52.0	79.0
	Kidney medulla			27.8	52.5
Ox	Kidney cortex (outer)			42.5	52.4
	Kidney cortex (inner)			42.8	52.8
	Kidney medulla			25.2	34.7

diesterase is suggested by the ability of the enzyme to differentiate completely between the optical enantiomorphs of the substrate.

Of the phosphodiesterases known to occur in tissues, the enzyme most resembles that which degrades adenosine 3':5'-cyclic phosphate, which also has an alkaline pH optimum and is activated by Mg²⁺. However, the distribution of the latter enzyme in rabbit and dog tissues is quite different from that observed in the present investigation, with the brain being many times more active than the kidney (Drummond & Perrott-Yee, 1961; Butcher & Sutherland, 1962). However, the situation may be complicated by the finding (Jard & Bernard, 1970) that in rat kidney adenosine 3':5'-cyclic phosphate can be broken down by two phosphodiesterases. It is also possible that the tissues may vary in the extent to which the extraction procedure solubilizes the present enzyme. The question of the substrate specificity and identification with or differentiation from other reported phosphodiesterases must await purification of the enzyme. This in turn will depend on the development of better ways for preparing the substrate in quantity and the ease with which activity can be determined. Substrate availability has severely limited any extensive kinetic studies in the present

investigation, and determinations of activity could only be done with concentrations of substrate that just saturated the enzyme.

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