The Diglyceride Kinase of Rat Cerebral Cortex

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1. Formation of phosphatidic acid by diglyceride kinase (EC 2.7.1.-) in the presence of ATP and Mg^{2+} was shown in a homogenate and subcellular fractions of rat cerebral cortex. 2. The kinase was activated by Mg^{2+} . Ca^{2+} activated to a smaller extent but was inhibitory in the presence of optimum concentration of Mg^{2+} . Activity was greatly increased in the presence of added 1,2-diglyceride. 3. Sodium deoxycholate markedly stimulated the reaction, but other detergents (Cutscum and Triton X-100) did not. 4. Diglyceride kinase was concentrated in the supernatant and microsomal fractions from rat cerebral cortex. The distribution of the kinase in the particulate fractions resembled that of acetylcholinesterase and 5'-nucleotidase. 5. The rate of phosphatidic acid synthesis by the diglyceride kinase route was much greater than reported rates for acylation of 3-glycerophosphate and was also very rapid in comparison with the rates of other steps in the synthesis of phosphoinositides. 6. Acetylcholine had no stimulatory effect on diglyceride kinase of isolated intact nerve-ending particles or of nerve-ending membranes obtained after osmotic shock.

Phosphatidic acid can be formed in brain by the acylation of lysophosphatidic acid, which is produced either by acylation of 3-glycerophosphate or by phosphorylation of monoglyceride (McMurray, Strickland, Berry & Rossiter, 1957; Pieringer & Hokin, 1962). Phosphatidic acid can also be synthesized by the phosphorylation of 1,2-diglyceride (Hokin & Hokin, 1959a). Incorporation of [^{32}P]P into phosphatidic acid of brain is stimulated by acetylcholine in tissue slices, in microsomes and in a fraction rich in nerve endings (Hokin & Hokin, 1958a,b; Durell & Sodd, 1964, 1966). To date two hypotheses have been advanced to explain this stimulation. These suggest that stimulation by acetylcholine occurs either at the level of diglyceride kinase (Redman & Hokin, 1964) or by activation of a phosphatidylinositol-inositolphosphohydrolase (Durell, Garland & Friedel, 1969). We report here some of the properties of the diglyceride kinase of rat cerebral cortex, its distribution in isolated subcellular fractions and the effects of acetylcholine on its activity.

MATERIALS

Reagents were obtained from the following sources: carrier-free [^{32}P]P_i, type PBS1, from The Radiochemical Centre (Amersham, Bucks., U.K.); 3-phosphoglycerate kinase, glyceraldehyde 3-phosphate dehydrogenase, aden-

osine deaminase and 3-phosphoglycerate from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany); ATP, AMP, NADH, bovine serum albumin, L-cysteine, tris, acetylthiocholine iodide and 5,5'-dithiobis-(2-nitrobenzoic acid) from Sigma (London) Chemical Co. Ltd. (London S.W.6, U.K.); *Clostridium perfringens* type A filtrate concentrate, as a source of phospholipase C, from Wellcome Research Laboratories (Beckenham, Kent, U.K.); acetylcholine chloride, ascorbic acid, deoxycholic acid and Folin-Ciocalteu Reagent from British Drug Houses Ltd. (Poole, Dorset, U.K.); Cutscum (iso-octylphenoxypolyethanol) from Kodak Ltd. (Kirkby, Lancs., U.K.); Triton X-100 (*p*-1,1,3,3-tetramethylbutylphenylpolyethoxyethanol) from Lennig Chemicals Ltd. (Jarrow, Co. Durham, U.K.); Ficoll from Pharmacia (Uppsala, Sweden). All organic solvents (analytical grade) were from Fisons Scientific Apparatus Ltd. (Loughborough, Leics., U.K.).

METHODS

Protein. This was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Total phosphate. This was measured by the method of King (1932).

Carboxylic esters. These were determined by the method of Stern & Shapiro (1953).

[γ - ^{32}P]ATP. Terminally-labelled ATP was prepared enzymically by the method of Glynn & Chappell (1964). After chromatographic purification the 0.25 M-HCl eluate was neutralized with solid tris to pH 7.4 and stored at -20°C until required.

L-1,2-Diglyceride. This lipid was prepared from purified ox brain phosphatidylcholine by the action of phospholipase C and purified as described by Gurr, Brindley &

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Hübscher (1965). To minimize conversion of 1,2-diglyceride into its 1,3-isomer the L-1,2-diglyceride was dissolved in hexane, divided into small portions and stored at -20°C until needed. The yield was assessed by an ester determination.

Preparation of brain subcellular fractions. For each fractionation, grey matter (2.5–4.0g/experiment) from four to six male albino rats (300g) was suspended in 4 ml of 0.32M-sucrose (adjusted to pH 7.4 with tris)/g of tissue and homogenized in a stainless-steel tube with a Teflon pestle (radial clearance 0.10–0.15 mm) rotated at 600 rev./min. Six complete strokes were used during 1 min of homogenization and this was repeated after 1 min. The homogenate was diluted to 10% (w/v) with 0.32M-sucrose and primary subcellular fractions were obtained as described by De Robertis, Pellegrino de Iraldi, Rodriguez de Lores Arnaiz & Salganicoff (1962) and Kataoka & De Robertis (1967). The crude 'mitochondrial' fraction was subfractionated under iso-osmotic conditions by using a discontinuous gradient of Ficoll (13%, 8% and 3%, w/v) in 0.32M-sucrose (Alberici, Rodriguez de Lores Arnaiz & De Robertis, 1965). Nerve-ending membranes were obtained by the method of Rodriguez de Lores Arnaiz, Alberici & De Robertis (1967). The fraction named M_1 1.0 which mainly contained nerve-ending membranes, was used in our experiments.

Assay of L-1,2-diglyceride kinase (ATP-1,2-diglyceride phosphotransferase, EC 2.7.1.-) activity. The conditions resembled those described by Prottey & Hawthorne (1967) for pancreatic diglyceride kinase. The activity was determined by measuring the incorporation of ^{32}P from [γ - ^{32}P]ATP into phosphatidic acid. The quantity of phosphatidic acid synthesized was calculated from the specific radioactivity of the ATP, assuming that only the terminal phosphate was incorporated into the lipid.

The basic system (in a total volume of 1 ml) contained: L-1,2-diglyceride (10 mM), sodium deoxycholate (0.1%, w/v), potassium phosphate buffer, pH 7.4 (50 mM), MgCl_2 (10 mM), KF (10 mM), [γ - ^{32}P]ATP (5 mM; specific radioactivity about 600 c.p.m./nmol) and enzyme protein (approx. 2 mg of particulate fractions or 0.6 mg of soluble fraction).

Appropriate samples of a solution of L-1,2-diglyceride in hexane were placed in test-tubes and the solvent was evaporated under a stream of N_2 with the tubes cooled in ice. Deoxycholate was added and the contents of the tubes were mixed for 30 s. In some assays deoxycholate was omitted: this is indicated where appropriate. All other components of the system, with the exception of the enzyme preparation, were added and the mixture incubated at 37°C for 10 min. The enzyme preparation was then added, the tubes were thoroughly mixed for 15 s and incubation was continued for 10 min. If the 10 min pre-incubation was omitted the system showed an increased requirement for diglyceride. Reactions were stopped, lipids were extracted and the radioactivity of the total lipids was measured as was described by Prottey & Hawthorne (1967).

Analysis of the extracted lipids by t.l.c. (Skipski, Peterson & Barclay, 1964) showed that more than 90% of the total radioactivity migrated with a phosphatidic acid marker.

Assay of marker enzymes. Several enzymes were assayed as markers to indicate the distribution of various

cell components in the different subcellular fractions obtained.

(i) Cytochrome oxidase (cytochrome c - O_2 oxidoreductase, EC 1.9.3.1) activity was assayed by the method of Wharton & Tzagoloff (1967) except that 0.1% (w/v) Triton X-100 was included (P. H. Cooper, personal communication).

(ii) 5'-Nucleotidase (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5) activity was measured by the method of Belfield & Goldberg (1968), in which interference from other phosphatases is minimized by pre-incubation with an excess of 2-glycerophosphate, and then AMP breakdown is assayed by an enzymic measurement of adenosine release.

(iii) Acetylcholinesterase (acetylcholine acetyl-hydrolase, EC 3.1.1.7) activity was determined at 37°C by the method of Ellman, Courtney, Andres & Featherstone (1961).

(iv) Lactate dehydrogenase (L-lactate-NAD oxidoreductase, EC 1.1.1.27) activity was measured by the method of Johnson (1960) as modified by Harwood & Hawthorne (1969).

RESULTS

Properties of diglyceride kinase in cerebral-cortex homogenate. These studies were made to establish the assay conditions for diglyceride kinase in the different subcellular fractions from rat cerebral cortex. Results for homogenate and crude mitochondrial fraction are plotted together on some of the figures. In some cases (Fig. 2) results are presented as percentages of the maximum activity observed, to facilitate comparisons between different experiments.

Diglyceride and ATP requirements. The effect of varying the concentration of L-1,2-diglyceride on phosphatidic acid formation in homogenates and crude mitochondrial fractions is shown in Fig. 1(a). Additions of 8–12 mM-L-1,2-diglyceride stimulated the synthesis of phosphatidic acid to a maximum rate of about eight times that obtained in the absence of exogenous diglyceride.

With ATP, activity reached a maximum at 5 mM (Fig. 1b) and decreased above this concentration.

Reaction rate and optimum enzyme concentration. The time-course of the enzyme reaction (Fig. 1c) was linear for 15 min when the phosphatidate synthesis reached a maximum. After this time, the labelling of the phosphatidic acid decreased. Hokin, Yoda & Sandhu (1966) found that the diglyceride kinase reaction was linear for at least 15 min.

Fig. 1(d) shows that the rate was proportional to enzyme concentration in the range 0–2.3 mg of protein.

Effect of pH. The homogenate and crude mitochondrial fractions showed a peak in diglyceride kinase activity at pH 7.4 (Fig. 2a). In some experi-

Fig. 1. Properties of cerebral diglyceride kinase. The assay system is described in the Methods section. (a) Effect of diglyceride concentration on phosphatidic acid synthesis: ●, homogenate; ○, crude mitochondrial fraction. (b) Effect of ATP concentrations on phosphatidic acid synthesis. (c) Time-course of the kinase reaction. (d) Protein concentration-activity curve for phosphatidic acid synthesis.

ments a second smaller peak at about pH 8.0 was observed. In these experiments this shoulder might have been contributed by soluble enzyme, since pH-activity curves performed with the supernatant fraction emphasized the peak at pH 8.0. In all of the present studies the activity was measured at pH 7.4.

Effect of bivalent cations. The activity of the diglyceride kinase was Mg^{2+} -dependent, the highest rates of phosphatidic acid synthesis being observed at 5–10 mM-magnesium chloride (Fig. 2b). In the presence of either calcium chloride (10 mM) or both calcium chloride (10 mM) and magnesium chloride (10 mM) the phosphatidic acid synthesis was only 22% of that in the presence of 10 mM- Mg^{2+} .

Effect of fluoride. Maximum phosphatidic acid synthesis was observed in the presence of 10–20 mM-

fluoride (Fig. 2c), which probably acted as an inhibitor of adenosine triphosphatases (Prottey & Hawthorne, 1967) and phosphatidate phosphatase (Coleman & Hübscher, 1962).

Effect of detergents. The effect of varying the concentration of deoxycholate is shown in Fig. 2(d). A sharp optimum concentration was found at 0.1% (w/v) for both the homogenate and a crude mitochondrial fraction. The same sort of curve was obtained with a brain microsomal fraction by Hokin & Hokin (1959a). The non-ionic detergent Cutscum at concentrations of 0.1–1.0% (w/v) completely inhibited phosphatidic acid synthesis. Triton X-100 (0.1%, v/v) did not affect the synthesis of phosphatidic acid. In the presence of deoxycholate (0.1%, w/v), 0.1% Triton X-100 caused an inhibition of 40%.

Fig. 2. Properties of cerebral diglyceride kinase. The basic assay system was used. ●, Homogenate; ○, crude mitochondrial fraction. (a) Effect of pH on the kinase. (b) Effect of Mg^{2+} on the kinase. (c) Effect of fluoride on the kinase. (d) Effect of deoxycholate concentration on phosphatidic acid synthesis.

Localization of diglyceride kinase in rat cerebral cortex. The results are summarized in Tables 1 and 2. Diglyceride kinase activity was observed in both soluble and particulate fractions. A comparison of the percentages of the diglyceride kinase recovered in the soluble fractions in the two sets of experiments suggests that about 10% of the total activity was solubilized during the procedure of cell fractionation. However, repeated washing of the mixed particulate fraction with 0.32M-sucrose did not release enzymic activity from this fraction.

In several experiments the crude 'mitochondrial' fraction was subfractionated to give fractions rich in myelin, nerve endings and mitochondria. In these experiments the recoveries of diglyceride kinase activity were very low (about 30%), but better recoveries were achieved when deoxycholate was omitted from the assay system (Table 2). The

Table 1. Diglyceride kinase activity in total particulate and supernatant fractions of rat cerebral cortex

The assay system was that described in the Methods section and contained deoxycholate. Values are the means of four experiments $\pm$ s.d. The fractions were obtained by centrifugation of the rat cerebral-cortex homogenate at 6×10^6 g-min. Percentages are related to the total recovered activity or protein. Relative specific activity (RSA) is given for each fraction as percentage of the recovered activity divided by percentage of the recovered protein.

	Protein (%)	Diglyceride kinase	
		(%)	(RSA)
Total particulate	80.0 ± 2.7	77.7 ± 4.0	0.97
Supernatant	20.0 ± 2.7	22.3 ± 4.0	1.12
Recovery (% of homogenate)	92.9 ± 5.2	101.5 ± 13.0	—

Table 2. *Subcellular distribution of protein, diglyceride kinase, cytochrome c oxidase, 5'-nucleotidase, acetylcholinesterase and lactate dehydrogenase in rat brain cortex*

Results are given as in Table 1 and S.D. values are quoted where more than three experiments were done. Percentages are related to the total recovered activity or protein. Numbers in parentheses indicate numbers of experiments. Abbreviations: DOC, deoxycholate; RSA, relative specific activity.

		Diglyceride kinase			
		With DOC		Without DOC	
	Protein (%) (11)	(%) (8)	(RSA)	(%) (3)	(RSA)
Primary fractions					
Nuclear	7.2 ± 1.4	5.9 ± 1.2	0.82	5.9 ± 1.8	0.82
Crude mitochondria	48.3 ± 4.8	37.4 ± 6.4	0.74	31.3 ± 0.0	0.65
Mic ₂₀	12.6 ± 3.1	11.0 ± 2.8	0.87	7.7 ± 2.7	0.61
Mic ₁₀₀	10.8 ± 2.9	14.0 ± 1.8	1.30	11.8 ± 3.6	1.09
Supernatant	21.2 ± 1.6	31.9 ± 5.0	1.50	43.2 ± 6.6	2.04
Recovery (% of homogenate)	89.6 ± 7.5	80.0 ± 9.1	—	94.5 ± 11.6	—
Submitochondrial fractions	(2)			(2)	
Myelin (A)	18.0	—	—	21.5	1.20
Nerve endings (B)	26.3	—	—	27.8	1.06
Nerve endings (C)	18.9	—	—	17.2	0.91
Mitochondria (D)	36.8	—	—	33.5	0.91
Recovery (% of crude mitochondria)	96.6	—	—	105.2	—
Absolute values for homogenate	(15)	(12)		(3)	
(mg/g of fresh tissue)	119.7 ± 13.2	—		—	
(nmol/min per mg of protein)	—	13.5 ± 3.0		1.94 ± 0.02	
Cytochrome c oxidase		5'-Nucleotidase		Acetylcholinesterase	
(%) (3)	(RSA)	(%) (3)	(RSA)	(%) (4)	(RSA)
5.4 ± 1.8	0.75	6.6 ± 2.9	0.92	2.7 ± 0.2	0.38
83.0 ± 6.2	1.72	46.4 ± 7.7	0.96	48.8 ± 10.9	1.01
9.4 ± 4.6	0.75	14.8 ± 2.8	1.17	21.1 ± 1.8	1.68
2.2 ± 0.9	0.20	22.2 ± 8.6	2.06	20.3 ± 8.1	1.88
0.0	0.0	10.1 ± 3.7	0.48	6.6 ± 2.9	0.31
99.8 ± 23.2	—	110.5 ± 16.5	—	92.0 ± 10.3	—
(2)		(2)		(2)	
3.5	0.19	18.3	1.02	15.3	0.85
5.3	0.20	38.8	1.47	36.4	1.38
19.2	1.02	24.5	1.30	25.6	1.35
72.0	1.96	18.3	0.50	27.7	0.62
78.9	—	83.0	—	87.5	—
(3)		(3)		(4)	
58.0 ± 10.5		13.1 ± 4.5		97.7 ± 14.4	
Lactate dehydrogenase					
(%) (2)	(RSA)			(%) (2)	(RSA)
3.3	0.46			3.3	0.46
27.8	0.58			27.8	0.58
8.6	0.68			8.6	0.68
3.0	0.28			3.0	0.28
56.4	2.66			56.4	2.66
82.2	—			82.2	—
(2)				(2)	
17.0	0.94			17.0	0.94
30.8	1.17			30.8	1.17
25.5	1.35			25.5	1.35
27.6	0.75			27.6	0.75
58.5	—			58.5	—
(2)				(2)	
0.97				0.97	

deoxycholate concentration-activity curves for the homogenate and crude 'mitochondrial' fraction both showed very sharp peaks and it is possible that the poor recoveries when deoxycholate was present were due to slight deviations from the optimum assay conditions, possibly owing to changes in the deoxycholate/membrane protein ratio.

The distribution of diglyceride kinase activity was compared with the distributions of the 'marker' enzymes cytochrome oxidase (mitochondria), lactate dehydrogenase (cytosol), 5'-nucleotidase and

acetylcholinesterase (both present in cell surface membranes in certain tissues; Michell & Hawthorne, 1965; Lapetina, Soto & De Robertis, 1967). The total activities of diglyceride kinase were highest in the crude mitochondrial and soluble fractions, each containing about one-third of the total activity of the homogenate. The soluble fractions had the highest specific activity for diglyceride kinase, but contained little 5'-nucleotidase and acetylcholinesterase. When the distributions in the particulate fractions of the remaining activity for each enzyme were then compared, it was apparent that the

distributions of diglyceride kinase, 5'-nucleotidase and acetylcholinesterase were quite similar, with their highest activities in a microsomal fraction (Mic_{100}) which is relatively free of nerve-ending particles (Kataoka & De Robertis, 1967). These three enzymes clearly were distributed differently from the other two markers.

When deoxycholate was omitted from the assay system, the proportion of the total diglyceride kinase activity apparently present in the soluble fraction was raised, almost certainly due to lack of access of the added diglyceride to the diglyceride kinase of the particulate fractions in the absence of detergent. In the submitochondrial fractions satisfactory recoveries of diglyceride kinase were recorded only in the absence of deoxycholate. Under these conditions the specific activity was higher in the fractions of lower density (myelin and nerve endings) and low in the subfractions containing mitochondria, but the diglyceride kinase could not be clearly assigned to any single intracellular site.

Effect of acetylcholine on diglyceride kinase. Hokin & Hokin (1958b) showed that under conditions suitable for oxidative phosphorylation acetylcholine stimulated the incorporation of $[^{32}P]P_i$ into the phosphatidic acid of all the cytoplasmic particulate fractions from guinea-pig brain, with the greatest stimulation in a microsomal fraction.

In brain slices acetylcholine stimulated the incorporation of $[^{32}P]P_i$ into both phosphatidylinositol and phosphatidic acid (Hokin & Hokin, 1955, 1958a). Hokin & Hokin (1959b) considered that in their experiments synthesis by the diglyceride kinase route was stimulated, with no change in the rate of acylation of glycerophosphate. This effect was dependent on the presence of intact subcellular structures and was abolished by freezing, under hypo-osmotic conditions or in the presence of deoxycholate.

In more recent experiments it was shown that the main site of stimulated phosphatidic acid labelling from $[^{32}P]P_i$ is in intact nerve endings, the label

presumably passing through a pool of ATP inside the nerve endings before being used for phosphatidic acid synthesis (Durell & Sodd, 1966).

In our experiments, diglyceride kinase preparations of homogenate and subcellular fractions obtained from rat cerebral cortex were assayed in the presence of acetylcholine (0.1 mM) and eserine (0.1 mM). In these determinations deoxycholate was omitted from the basic assay procedure to preserve the integrity of the fractions. The results (Table 3) show no increase in activity as measured by the formation of $[^{32}P]$ phosphatidic acid.

DISCUSSION

Properties of brain diglyceride kinase. An enzyme catalysing reaction (1) (Scheme 1) was first detected in deoxycholate extracts of brain microsomes by Hokin & Hokin (1959a), who had shown that heterogeneous 'mitochondrial' and 'microsomal' fractions from brain catalysed phosphatidic acid synthesis by a pathway other than the acylation of glycerophosphate. Diglyceride kinase has since been described in erythrocyte 'ghosts' (Hokin & Hokin, 1963), polymorphonuclear leucocytes (Sastry & Hokin, 1966), pancreas (Prottey & Hawthorne, 1967), avian salt-gland (Hokin & Hokin, 1964) and particulate preparations from *Escherichia coli* (Pieringer & Kummer, 1965). In the present work optimum conditions for the assay of the brain enzyme have been established and its intracellular location investigated.

The diglyceride kinase of brain is a typical phosphokinase, requiring Mg^{2+} at a concentration of about 10 mM. This requirement is similar to that of the pancreatic diglyceride kinase (Prottey & Hawthorne, 1967), and is considerably higher than the concentrations used by Hokin & Hokin (1959a) and by Hokin *et al.* (1966). Ca^{2+} can partially substitute for Mg^{2+} in the brain diglyceride kinase, as it also can for brain diphosphoinositide kinase (Kai, Salway & Hawthorne, 1968). At the optimum ATP concentration (5 mM) the Mg^{2+} /ATP concentra-

Table 3. *Effect of acetylcholine on diglyceride kinase activity in subcellular fractions from rat cerebral cortex*

The results are expressed as the ratio of diglyceride kinase activity in the presence of added acetylcholine to the activity in its absence. Diglyceride kinase activity was assayed as described in the Methods section, but without deoxycholate and with the addition of 0.1 mM-acetylcholine and 0.1 mM-eserine.

Expt. no. ...	1	2	3	4	5
Homogenate	1.01	0.80	—	—	—
Crude mitochondria	1.09	—	—	—	—
Microsomes (Mic_{100})	0.99	0.96	—	—	—
Supernatant	0.97	0.82	—	—	—
Nerve endings (B)	0.98	1.03	—	—	—
Mitochondria (D)	1.04	—	—	—	—
Nerve-ending membranes (M_1 1.0)	—	—	0.83	0.50	0.76

Scheme 1. Hypothetical scheme of reactions involved in the breakdown and resynthesis of phosphatidylinositol in response to acetylcholine (Redman & Hokin, 1964; Durell *et al.* 1969).

tion ratio was 2:1, again the same as for the pancreatic phosphorylation of diglyceride (Prottey & Hawthorne, 1967). No ATP optimum can be calculated from the experiments of Hokin & Hokin (1959a) and Hokin *et al.* (1966), since a system for continuous generation of ATP from carbamoyl phosphate was used in their assay method.

The pH optimum of the brain system is 7.4. This is the same as that used by Hokin *et al.* (1966) and by Prottey & Hawthorne (1967) for the pancreatic kinase. The early experiments of Hokin & Hokin (1959a) were at pH 6.5, which may at least partially explain the very low rates of phosphatidic acid synthesis in their experiments (see Table 4). We have confirmed that diglyceride kinase is stimulated by addition of deoxycholate (Hokin & Hokin, 1959a; Hokin *et al.* 1966), but neither of the neutral detergents that we tested (Triton X-100 and Cutseum) caused any increase in phosphatidic acid synthesis. The way in which deoxycholate acts in this system is not completely clear, but it is probably acting by solubilizing the highly hydrophobic substrate and also by disrupting membranes and giving improved access of substrate to membrane-bound kinase. This dual interpretation is supported by the lower

degree of stimulation observed with the soluble portion of the kinase activity. The dependence of diglyceride kinase on the addition of exogenous diglyceride was very clear in brain, but not complete as in a pancreas homogenate (Prottey & Hawthorne, 1967). The effect on the activity of brain diglyceride kinase of using diglyceride derived from sources other than ox brain phosphatidylcholine was not investigated: no experiments have yet been reported in which arachidonoylstearyl diglyceride (as would be derived from one of the major species of animal phosphatidylinositol) has been used as a substrate for diglyceride kinase from any source, so the rates of synthesis until now must be regarded as minimum values.

Subcellular distribution of diglyceride kinase. Diglyceride kinase was present in quite high activity in all the subcellular fractions isolated from rat brain, with no obvious localization at any single subcellular site. The highest specific activities were in the soluble fraction and in a microsomal fraction relatively free of nerve-ending particles (Mic₁₀₀). However, comparison of the distributions of two putative surface membrane markers (5'-nucleotidase and acetylcholinesterase) with that of the

Table 4. *Enzymes of phosphoinositide synthesis in brain*

Activity	Source	Preparation	Detergent	Rate (nmol/ h per mg of protein)	Reference
Diglyceride kinase	Rat cerebral cortex	Homogenate	None	116	Present work
	Guinea-pig brain	Particulate fraction	Deoxycholate Deoxycholate	810 ~100	Present work Hokin <i>et al.</i> (1966)
Acylation of 3-glycerophosphate	Rat brain	9500g pellet fraction + boiled supernatant fraction	None	6.4	Mårtensson & Kanfer (1968)
CTP-phosphatidate cytidyltransferase	Embryonic chick brain	12000g fraction	Cutscum	0.090	Petzold & Agranoff (1967)
CDP-diglyceride-inositol phosphatidyltransferase	Rat brain	Homogenate	None	24.9	Salway, Harwood, Kai, White & Hawthorne (1968)
Phosphatidylinositol kinase	Rat brain	Homogenate	Cutscum	8.9	Salway <i>et al.</i> (1968)
Diphosphoinositide kinase	Rat brain	Homogenate	None	16.8	Kai <i>et al.</i> (1968)

kinase in the particulate fractions showed that they were clearly similar, though not identical. This is a similar situation to that reported for phosphatidylinositol kinase of brain (Kai, White & Hawthorne, 1966; Harwood & Hawthorne, 1969), except that for phosphatidylinositol kinase clear evidence for localization in plasma membrane is available, at least for liver (Michell, Harwood, Coleman & Hawthorne, 1967). As pointed out by Kai *et al.* (1966), results based on comparisons with putative marker enzymes must be interpreted very cautiously when a tissue as complex as brain is being considered. However, diglyceride kinase has also been reported as a component of the surface membrane of the erythrocyte, (Hokin & Hokin, 1963); and it may well be that a considerable proportion of the particulate diglyceride kinase in brain is located in cell surface membrane.

Rates of synthesis for phosphatidic acid and the phosphoinositides. Hawthorne & Kai (1970) have compared the rates so far recorded for several reactions involved in the metabolic routes leading to these lipids in rat brain. In Table 4 these values are presented, together with the rates of synthesis of phosphatidic acid via the diglyceride kinase route and via acylation of glycerophosphate. Clearly diglyceride kinase represents an important route for the synthesis of phosphatidic acid (step 1 in Scheme 1) and ultimately of all the inositol lipids. It seems unlikely that this will be a rate-limiting, and thus controlling, step on the pathway.

Effect of acetylcholine on phosphatidic acid labelling in brain. The site of this stimulation was

localized to intact nerve-ending particles present in the subcellular fractions (Durell & Sodd, 1966). In tissue slices this phosphatidic acid is further converted into phosphatidylinositol. Studies of this effect in brain slices by using precursors for different parts of the lipid molecule demonstrate that the diglyceride moiety of the phosphatidylinositol does not show the stimulated turnover, and thus that the stimulated turnover presumably occurs via the diglyceride kinase pathway, with as substrate diglyceride produced by the hydrolysis of phospholipid (Hokin & Hokin, 1958a). Redman & Hokin (1964) suggested that acetylcholine acts by a stimulation of the diglyceride kinase (step 1 in Scheme 1). However, direct stimulation of the diglyceride kinase of brain assayed under well-characterized conditions has never been demonstrated. Durell *et al.* (1969) have produced information suggesting that hydrolysis of phosphatidylinositol (step 4 in Scheme 1) may be the stimulated step in the pathway. Lunt, Canessa & De Robertis (1971) have since shown localization of the acetylcholine stimulation of phosphatidylinositol turnover in a fraction of the brain phosphatidylinositol that is tightly bound to a brain proteolipid. This proteolipid shows many of the suggested properties of an acetylcholine receptor molecule and responds conformationally to the addition of cholinergic neuroactive agents (De Robertis, 1971). In view of all these suggestions we have studied the effect of acetylcholine on one of these enzymes, the diglyceride kinase. Measurements of activity were made both in intact nerve-endings and in nerve-ending

membranes isolated after osmotic shock. The results (Table 3) showed that there is no direct stimulation by acetylcholine of diglyceride kinase either on the outer or on the inner face of the membrane at the surface of the synaptic region of the neurone. Another tissue in which acetylcholine stimulates turnover of phosphatidic acid and phosphatidylinositol is mammalian pancreas, but addition of acetylcholine to homogenates of this tissue does not stimulate the diglyceride kinase (White & Hawthorne, 1970). The lack of effect of acetylcholine on diglyceride kinase in both brain and pancreas seems to eliminate one of the suggested mechanisms for acetylcholine stimulation of phospholipid turnover. Further work is still needed before the true mechanism of this effect becomes clear.

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