Factors Controlling the Activities of Phosphatidate Phosphohydrolase and Phosphatidate Cytidylyltransferase

THE EFFECTS OF CHLORPROMAZINE, DEMETHYLIMIPRAMINE, CINCHOCAINE, NORFENFLURAMINE, MEPYRAMINE AND MAGNESIUM IONS

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1. Microsomal membranes from rat liver were incubated with ATP, CoA, Mg^{2+} , [^{14}C]palmitate, F^- and *sn*-glycerol 3-phosphate in order to label them with [^{14}C]phosphatidate. These membranes were isolated and used in a second incubation in which [3H]CTP was present, and the simultaneous synthesis of [^{14}C]diacylglycerol and [3H]CDP-diacylglycerol was measured. 2. The addition of phosphatidate phosphohydrolase, which had been partially purified from the particle-free supernatant, supplemented the activity of the endogenous phosphohydrolase, but it did not alter the rate of CDP-diacylglycerol formation. 3. Adding EDTA inhibited phosphatidate cytidylyltransferase activity and stimulated the activity of the phosphohydrolases by removing excess of Mg^{2+} . 4. Increasing the concentration of Mg^{2+} , norfenfluramine or chlorpromazine in the assay system stimulated cytidylyltransferase activity, but decreased the activities of both phosphohydrolases. 5. The mechanism for the stimulation of cytidylyltransferase activity by the cationic drugs and Mg^{2+} was investigated with emulsions of phosphatidate and the microsomal fraction of rat liver. 6. There was a threshold concentration of about 5 mM- $MgCl_2$ below which no cytidylyltransferase activity was detected in the presence or absence of norfenfluramine. Just above this threshold concentration norfenfluramine stimulated cytidylyltransferase activity, but this stimulation disappeared as the Mg^{2+} concentration was raised to its optimum of 20 mM. Norfenfluramine therefore partially replaced the bivalent-cation requirement. 7. At 30 mM- $MgCl_2$ amphiphilic cationic drugs inhibited cytidylyltransferase activity at relatively high concentrations in a non-competitive manner with respect to phosphatidate. 8. The implications of these results are discussed with respect to the regulation of the synthesis of the acidic phospholipids compared with the synthesis of phosphatidylcholine, phosphatidylethanolamine and triacylglycerol.

Drugs such as the phenothiazine neuroleptics, tricyclic antidepressants, local anaesthetics, fenfluramine and its derivatives, propranolol and other β -adrenergic-blocking agents and some narcotic drugs lead to profound changes in glycerolipid metabolism in a variety of tissues. These compounds all contain a hydrophobic region and a primary or substituted amine which can bear a positive charge. They can therefore be classified as amphiphilic cations. These drugs all decrease the rates of synthesis of triacylglycerol and the zwitterionic phospholipids, phosphatidylcholine and phosphatidylethanolamine. At the same time phosphatidate and other acidic phospholipids such as CDP-diacylglycerol, phosphatidylinositol and phosphatidylglycerol accumulate (for references, see Eichberg & Hauser, 1974, 1975; Allan & Michell, 1975; Brindley & Bowley, 1975a,b; Michell *et al.*, 1976; Abdel-

Latif & Smith, 1976). These amphiphilic cations have been shown to inhibit the activity of phosphatidate phosphohydrolase (EC 3.1.3.4) (Brindley & Bowley, 1975a,b) and this action could explain many of their effects on lipid synthesis (Eichberg & Hauser, 1974; Brindley *et al.*, 1975).

It has been proposed that the inhibition of phosphatidate phosphohydrolase might involve an interaction between phosphatidate and the positively charged drugs (Brindley & Bowley, 1975a). The same complex would presumably also act as the substrate for phosphatidate cytidylyltransferase (EC 2.7.7.41). If the cationic drugs are to redirect the flux of substrate towards the synthesis of the acidic phospholipids, then the activity of cytidylyltransferase would have to be stimulated or at least be fairly resistant to inhibition by these compounds. The present experiments investigate this hypothesis and also

examine the effects of amphiphilic cations on the relative rates of conversion of phosphatidate into diacylglycerol and CDP-diacylglycerol.

Materials and Methods

Animals

Male Wistar rats (200–250 g) were obtained from the University of Nottingham Joint Animal Breeding Unit, Sutton Bonington, Leics., U.K. They were allowed free access to food and water before the experiments.

Materials

Unless stated to the contrary, cofactors, substrates and reagents were prepared or purchased as described previously (Sánchez *et al.*, 1973; Mangiapane *et al.*, 1973; Brindley & Bowley, 1975a). CTP (Na^+ salt, from yeast) was purchased from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K., [^3H]CTP (ammonium salt) from The Radiochemical Centre, Amersham, Bucks., U.K., and CDP-diacylglycerol from Serdary Research Laboratories, 1643 Kathryn Drive, London, Ont., Canada.

Preparation of cell fractions

Cell fractions were prepared from rat liver essentially as described by Mangiapane *et al.* (1973). However, the livers were perfused *in situ* with ice-cold 0.9% NaCl when the particle-free supernatant was to be used as a source of enzyme.

Preparation of phospholipase D (EC 3.1.4.4)

The method was essentially that of Davidson & Long (1958), but white celery was used for the preparation. Celery stalks (300 g) were homogenized in 450 ml of water in a blender. The homogenate was filtered through cheesecloth and centrifuged at 13000g for 30 min. The supernatant was taken as a source of crude enzyme and used immediately.

Preparation of partially purified phosphatidate phosphohydrolase

The soluble phosphatidate phosphohydrolase obtained from the particle-free supernatant of perfused rat livers was purified by the method of Hosaka *et al.* (1975). The protein obtained after the $(\text{NH}_4)_2\text{SO}_4$ precipitation was dialysed overnight at 4°C against 0.3 M-sucrose containing 0.5 mM-dithiothreitol immediately before use. This gave an approximately 6-fold increase in the specific activity compared with that of the particle-free supernatant.

Preparation of membrane-bound [^{14}C]phosphatidate

[^{14}C]Phosphatidate was synthesized from [^{14}C]palmitate on the microsomal membranes of rat liver as described by Brindley & Bowley (1975a). The reaction was stopped after 30 min at 37°C by cooling the reaction mixture in an ice bath.

The mixture was layered over 0.3 M-sucrose containing 0.5 mM-dithiothreitol and centrifuged for 90 min at 4°C and 76000g ($r_{\text{av.}} = 7.62$ cm). The pellets which contained the newly synthesized phosphatidate were resuspended in fresh sucrose/dithiothreitol medium. Analysis of the [^{14}C]-labelled lipid by t.l.c. (Brindley & Bowley, 1975a) showed that approx. 87% of the esterified [^{14}C]palmitate was present in phosphatidate, with approx. 8% being divided equally between diacylglycerol and triacylglycerol.

Preparation of potassium phosphatidate

Phosphatidate was prepared by hydrolysis of phosphatidylcholine by phospholipase D. Phosphatidylcholine from egg yolk was purified by chromatography on aluminium oxide (Singleton *et al.*, 1965) and eluted with chloroform/methanol (9:1, v/v). It was then treated with the phospholipase D for 5 days at room temperature (about 22°C) essentially as described by Agranoff & Suomi (1963). The ether phase was removed and the aqueous phase plus the interface were then extracted three times with chloroform. The combined chloroform and ether phases were evaporated to dryness under reduced pressure. The residue was applied in chloroform to a silicic acid column and neutral lipids were eluted with chloroform. The phosphatidate was then eluted with chloroform/methanol (93:7, v/v) and the solvent evaporated under reduced pressure. The product was converted into potassium phosphatidate by chromatography on Chelex resin (K^+ form; Renkonen, 1969). Emulsions of phosphatidate (15 $\mu\text{mol}/\text{ml}$) were prepared after the removal of solvent by sonication for about 2 min in water at 22 kHz under an atmosphere of N_2 .

Assay of phosphatidate cytidyltransferase activity by using emulsions of phosphatidate

The method of assay was similar to that described by Carter & Kennedy (1966). Each assay contained in a final volume of 0.25 ml: 100 mM-Tris buffer adjusted to pH 7.4 with HCl, 2 mM-potassium phosphatidate, 1 mM-[^3H]CTP (2 $\mu\text{Ci}/\mu\text{mol}$), 5 mM-ATP, 1 mM-dithiothreitol, 6 mg of fatty acid-poor bovine serum albumin/ml and 0.3–0.5 mg of microsomal protein. The reaction was started by adding MgCl_2 as indicated and was stopped after 10 min at 37°C with 1.88 ml of chloroform/methanol (1:2, v/v). Lipids were extracted by the method of Hajra *et al.* (1968) as described by Brindley & Bowley (1975a). More than 95% of the [^3H]-labelled lipid co-chromatographed with authentic CDP-diacylglycerol on plates of silica gel 60 (Merck) developed in chloroform/methanol/acetic acid/acetone/water (10:2:2:4:1, by vol.) or chloroform/methanol/acetic acid/water (25:14:2:4, by vol.). The reaction rate was constant for 15 min of incubation and propor-

tional to the added microsomal protein up to a concentration of 1 mg/ml.

Simultaneous measurement of phosphatidate phosphohydrolase and phosphatidate cytidyltransferase with membrane-bound [^{14}C]phosphatidate as a substrate

The enzymes were assayed in endoplasmic-reticulum membranes which had been pre-labelled with [^{14}C]phosphatidate. Each assay contained in a final volume of 0.25 ml: 100 mM-Tris adjusted to pH 7.4 with HCl, 0.1 mM-EGTA, 2 mM- $[\text{^3H}]\text{CTP}$ (7 $\mu\text{Ci}/\mu\text{mol}$), 31 nmol of [^{14}C]phosphatidate (0.4 $\mu\text{Ci}/\mu\text{mol}$) bound to 440 μg of microsomal protein, and 6 mg of fatty acid-poor bovine serum albumin/ml. The reaction was stopped with 1.88 ml of chloroform/methanol (1:2, v/v) after incubation at 37°C for 7 min. Lipids were extracted by the method of Hajra *et al.* (1968) as described by Brindley & Bowley (1975a) by using three washes with synthetic top phase. Samples of the bottom phase (0.8 ml) were evaporated to dryness and dissolved in 150 μl of chloroform. Samples (100 μl) were analysed by t.l.c. on silica gel G. The plates were first developed for about 40% of their length with chloroform/methanol/acetic acid/acetone/water (10:2:2:4:1, by vol.). After being allowed to dry, the plates were then developed for their full length in hexane/diethyl ether/acetic acid (60:40:1, by vol.). The cytidyltransferase activity was defined as the incorporation of ^3H into CDP-diacylglycerol and that of phosphatidate phosphohydrolase as the ^{14}C incorporation into diacylglycerol. The rate of diacylglycerol synthesis was constant for at least 15 min, whereas that of CDP-diacylglycerol declined after 10 min.

Determination of protein

Protein concentrations were determined by a biuret method (Hübscher *et al.*, 1965).

Determination of radioactivity

Radioactivity was determined by liquid-scintillation counting as described by Brindley *et al.* (1976).

Experimental and Results

Effect of a variety of amphiphilic compounds on the activity of phosphatidate cytidyltransferase

If cationic drugs are to redirect glycerolipid synthesis towards the formation of CDP-diacylglycerol, then the cytidyltransferase should be fairly resistant to their inhibition. The effects of a series of amphiphilic cations on the activity of this enzyme were investigated by using phosphatidate emulsions with optimum concentrations of Mg^{2+} (30 mM) in the assay system. The concentrations of these drugs which were required to produce a 50% inhibition of the cytidyltransferase were higher than those which produced an equivalent inhibition of phosphatidate phosphohydrolase activity (Table 1). This is most marked for norfenfluramine and hydroxyethylnorfenfluramine. However, demethylimipramine and chlorpromazine were fairly effective inhibitors of the cytidyltransferase activity. The inhibition of phosphatidate cytidyltransferase activity was of a non-competitive type (Fig. 1), but it exhibited non-linear kinetics. This may be caused partly by the difficulties in defining the concentration of phosphatidate which is probably more related to the effective surface area of the phosphatidate emulsions than to its absolute concentration

Table 1. *Effect of various drugs and other compounds on the activities of phosphatidate cytidyltransferase, phosphatidate phosphohydrolase and glycerol phosphate acyltransferase*

Phosphatidate cytidyltransferase activity was measured by using an emulsion of phosphatidate in the presence of 30 mM- MgCl_2 as described in the Materials and Methods section. The concentrations of the compounds which were required to produce a 50% inhibition of this activity were calculated as described by Brindley & Bowley (1975a) and are accurate to within $\pm 10\%$. The numbers of rats used for these determinations are shown in parentheses. For comparative purposes the equivalent results obtained for glycerol phosphate acyltransferase and phosphatidate phosphohydrolase by Brindley & Bowley (1975a) are listed.

Compound	Concentration (mM) required to produce a 50% inhibition in the activity of:		
	Phosphatidate cytidyltransferase	Glycerol phosphate acyltransferase	Phosphatidate phosphohydrolase
Demethylimipramine	0.65 (3)	1.0 (4)	0.3 (4)
Chlorpromazine	1.2 (7)	1.0 (4)	0.2 (4)
Cinchocaine	2.6 (3)	2.6 (4)	0.4 (4)
Mepyramine	4.5 (3)	3 (4)	0.7 (4)
Norfenfluramine	10.5 (3)	11 (5)	0.9 (6)
Hydroxyethylnorfenfluramine	13 (3)	15 (5)	~0.4 (6)
Triton X-100	0.36 (4)	Not measured	Not measured
Clofenapate	0.65 (5)	1.6 (6)	6.8 (6)

Fig. 1. Inhibition of phosphatidate cytidyltransferase by chlorpromazine

Phosphatidate cytidyltransferase activity was measured by using emulsions of phosphatidate in the presence of 30mM-MgCl₂. The concentration of chlorpromazine that was added to the incubations is indicated as follows: 0mM (○), 0.6mM (△), 1.2mM (□), 2.0mM (▲), 3.0mM (●). The error bars indicate s.d. from three experiments. Two experimental points have been omitted from the Figure at 3mM-chlorpromazine and one at 2mM-chlorpromazine because of the problems of scale.

(Brindley & White, 1974). A strict kinetic interpretation is therefore not possible. However, the relative inhibition of the cytidyltransferase activity by chlorpromazine was independent of the phosphatidate concentration.

The inhibition by chlorpromazine was reversed when the membranes were centrifuged through 0.3M-sucrose containing 6mg of fatty acid-poor bovine serum albumin/ml and 0.5mM-dithiothreitol, and the rate of the inhibited reaction was constant with time up to 15min. These two observations support the conclusion that the inhibition was of a non-competitive rather than of an irreversible type. The inhibition of cytidyltransferase and of glycerol phosphate acyltransferase probably results from an interaction of the cationic drugs with the microsomal membranes. The concentrations of cationic drugs required for a 50% inhibition of these two enzymes (Table 1) showed highly significant correlation ($r = 0.99$; $P < 0.001$).

For comparison, the effects of a neutral detergent

(Triton X-100) and an amphiphilic anionic drug {clofenapate [4-(4'-chlorophenyl)phenoxyisobutyrate]} were tested. Both gave 50% inhibition of the cytidyltransferase activity at concentrations similar to those observed with demethylimipramine and chlorpromazine (Table 1). This demonstrates that the amphiphilic compounds do not need to bear a positive charge in order to act as inhibitors.

Effect of norfenfluramine, chlorpromazine and Mg²⁺ on the utilization of [¹⁴C]phosphatidate bound to the microsomal membranes of rat liver

The purpose of these experiments was to measure the effects of Mg²⁺ and amphiphilic cations on the simultaneous activities of phosphatidate phosphohydrolase and phosphatidate cytidyltransferase. The system chosen involved the use of [¹⁴C]phosphatidate bound to microsomal membranes as a substrate. This procedure has the advantage of providing a substrate which is probably more accessible for enzymic conversion (Brindley & White, 1974) and which has been widely used for studying the properties of phosphatidate phosphohydrolase. During the course of this work a paper also appeared on its use for studying the specificity of the cytidyltransferase (Holub & Piekarski, 1976). The experiments described in Fig. 2 use the activity of the phosphatidate phosphohydrolase and phosphatidate cytidyltransferase that were present in the same membranes as the [¹⁴C]phosphatidate.

Phosphatidate phosphohydrolase was also partially purified from the particle-free supernatant of rat liver and was added in some experiments. The addition of this phosphohydrolase did not significantly decrease the rate of synthesis of CDP-diacylglycerol in four experiments, even though the flux of phosphatidate to diacylglycerol was increased (see also Fig. 2). Adding norfenfluramine decreased the utilization of phosphatidate in the presence of soluble phosphohydrolase, owing to an inhibition of the combined phosphohydrolase activity (Fig. 2a). Fig. 2(a) shows the results from one experiment, since the absolute activity of the phosphohydrolase varied with different preparations. However, in three independent experiments the addition of 1.75mM-norfenfluramine caused a $62 \pm 8\%$ (s.d.) decrease in the utilization of phosphatidate. The membrane-bound and the soluble phosphohydrolase activities were inhibited to similar extents. At the same concentration of norfenfluramine the synthesis of CDP-diacylglycerol was increased by 3.1 ± 0.3 -fold. In the experiment shown in Fig. 2(a), the utilization of phosphatidate in the absence of the soluble phosphohydrolase was not significantly decreased, since the inhibition of the membrane-bound phosphohydrolase was counter-balanced by the increase in the activity of the cytidyltransferase.

Fig. 2. Effect of norfenfluramine and Mg^{2+} on the utilization of [^{14}C]phosphatidate bound to the microsomal membranes of rat liver

Membrane-bound [^{14}C]phosphatidate was incubated for 7 min in the presence and absence of norfenfluramine (a) and exogenous $MgCl_2$ (b). In the experiments described in (a) and (b) respectively, 300 and 270 μg of soluble protein from partially purified preparations of phosphatidate phosphohydrolase were added to some incubations. The enzyme activities are indicated by: Δ , total utilization of phosphatidate in the presence of soluble phosphatidate phosphohydrolase (i.e. total nmol of diacylglycerol plus CDP-diacylglycerol synthesized); $\blacktriangle$, total utilization of phosphatidate in the absence of soluble phosphatidate phosphohydrolase; $\circ$, activity of the soluble phosphatidate phosphohydrolase; $\bullet$, activity of the microsomal phosphatidate phosphohydrolase; $\square$, activity of phosphatidate cytidyltransferase in the presence of soluble phosphatidate phosphohydrolase; $\blacksquare$, activity of phosphatidate cytidyltransferase in the absence of soluble phosphatidate phosphohydrolase.

Similar results were obtained with chlorpromazine. In two experiments a maximum stimulation in the synthesis of CDP-diacylglycerol of 2.6- and 2.8-fold was obtained with 0.35 mM-chlorpromazine. This concentration of chlorpromazine decreased the average activity of the membrane-bound and soluble phosphohydrolases by 65% and 55% respectively.

Fig. 2(b) shows the effect of changing the concentration of Mg^{2+} on the relative utilization of phosphatidate by the phosphohydrolase and the cytidyltransferase. This effect is remarkably similar to that obtained with norfenfluramine and chlorpromazine. Addition of 20 mM- $MgCl_2$ gave a 3.3 ± 0.3 (s.d. from three experiments) -fold increase in the activity of the cytidyltransferase. The phosphohydrolase activities were completely inhibited by adding 20 mM- $MgCl_2$.

The [^{14}C]phosphatidate in the preparation contained some Mg^{2+} which was probably bound to it during the first incubation which was used to prelabel the membranes. This concentration of Mg^{2+} was inhibitory for the phosphohydrolase and a stimulation of about 3.3-fold was obtained by adding 2 mM-EDTA

(Fig. 3). Higher concentrations of EDTA inhibited when the effective concentration of Mg^{2+} required for the phosphohydrolase reaction became suboptimum. It is already known that a Mg^{2+} requirement is only observed for the phosphatidate phosphohydrolase acting on membrane-bound phosphatidate when the latter is extensively treated with EDTA (Mitchell *et al.*, 1971). Addition of EDTA in the present experiments completely inhibited the cytidyltransferase activity.

Interaction between Mg^{2+} and norfenfluramine on the activity of phosphatidate cytidyltransferase

The effects of Mg^{2+} and norfenfluramine in promoting the flux of phosphatidate towards CDP-diacylglycerol and away from diacylglycerol were similar (Fig. 2). Cationic drugs are known to compete with bivalent cations for binding sites in membranes and on acidic phospholipids (Mulé, 1969; Dawson & Hauser, 1970; Papahadjopoulos, 1970; Papahadjopoulos *et al.*, 1975). It was therefore decided to investigate the interaction of Mg^{2+} and norfenflur-

Fig. 3. Effect of EDTA on the activity of phosphatidate phosphohydrolase towards membrane-bound [^{14}C]phosphatidate

In Expt. 1 (●) 820 μg of protein from the particle-free supernatant was added to each incubation, whereas in Expt. 2 (▲) 200 μg of protein from a partially purified preparation of soluble phosphatidate phosphohydrolase was used. The rates of diacylglycerol synthesis in the absence of EDTA were 0.21 and 0.50 nmol/min in Expts. 1 and 2 respectively and these are given the value of 100 in the Figure.

Fig. 4. Effect of Mg^{2+} on the activity of phosphatidate cytidyltransferase

Phosphatidate cytidyltransferase activity was measured by using emulsions of phosphatidate as described in the Materials and Methods section. The requirement for Mg^{2+} with the microsomal fractions obtained from the livers of five different rats is indicated by: □, Expt. 1; ■, Expt. 2; ●, Expt. 3; ○, Expt. 4; ▲, Expt. 5.

amine on the activity of phosphatidate cytidyltransferase by using emulsions of phosphatidate.

Mg^{2+} requirement for the cytidyltransferase is shown in Fig. 4. There seems to be a threshold concentration of Mg^{2+} below which no enzyme activity is observed. Above this threshold the activity rises sharply and maximum velocities are obtained at about 20 mM- MgCl_2 . The requirement for Mg^{2+} appears to vary from experiment to experiment, and this is probably caused by the differences in the physical properties of the phosphatidate in different emulsions.

The interaction of norfenfluramine and Mg^{2+} is shown in Fig. 5, and these results were obtained in Expt. 5 (Fig. 4). At 5 mM- MgCl_2 a 5.3-fold stimulation of cytidyltransferase activity was obtained by adding 1.75 mM-norfenfluramine. As the concentration of Mg^{2+} was raised, the stimulation produced by norfenfluramine was decreased. At 20 mM- Mg^{2+} , which was an optimum concentration (Fig. 4), norfenfluramine produced no significant stimulation of CDP-diacylglycerol formation (Fig. 5). Fig. 5 contains the results from only one experiment, since the requirement for Mg^{2+} varied in different experiments (Fig. 4). However, the effect of norfenfluramine was similar in four independent experiments. In three experiments (including Expts. 1 and 3 of Fig. 4), there was no cytidyltransferase activity at 5 mM- MgCl_2 and the addition of norfenfluramine caused no stimulation of the activity.

Fig. 5. Effect of Mg^{2+} concentration on the stimulation of phosphatidate cytidyltransferase by norfenfluramine. Phosphatidate cytidyltransferase activity was measured by using phosphatidate emulsions as described in the Materials and Methods section. The effect of norfenfluramine on this activity is shown when the concentration of Mg^{2+} in the assay system was 5 mM (●), 7 mM (▲), 10 mM (■) and 20 mM (○).

Norfenfluramine only stimulated when the Mg^{2+} concentration was high enough to catalyse the synthesis of CDP-diacylglycerol. At 5 mM- $MgCl_2$ (Expt. 4, Fig. 4) the addition of 1.75 mM-norfenfluramine produced a 3.1-fold stimulation of cytidyltransferase activity. Similarly in Expt. 3 (Fig. 4) the same concentration of norfenfluramine stimulated the synthesis of CDP-diacylglycerol by 5.6-fold, but this was at 7 mM- $MgCl_2$.

Norfenfluramine therefore appears to be partially but not completely replacing the requirement for Mg^{2+} in the cytidyltransferase reaction. This explains the norfenfluramine-induced stimulation of CDP-diacylglycerol synthesis observed in Fig. 2(a) when membrane-bound phosphatide was used as a substrate. This is also confirmed by the observation that norfenfluramine (0.3–2.75 mM) only stimulated CDP-diacylglycerol synthesis by 1.2–1.3-fold when an optimum concentration of 20 mM- $MgCl_2$ was added to the incubations containing membrane-bound [^{14}C]phosphatide.

The amphiphilic cations might also increase the recovery of [3H]CDP-diacylglycerol by preventing the breakdown of this lipid. The enzymic hydrolysis of CDP-diacylglycerol has been demonstrated in *Escherichia coli* (Raetz *et al.*, 1976). There are indications that it may occur in mammalian tissues, since the recovery of 3H in [3H]CDP-diacylglycerol decreases when the incubation with membrane-bound phosphatide is extended beyond about 10 min (Holub & Piekarski, 1976; and present work). Adding norfenfluramine to this incubation system does not significantly prevent this decrease in recovery of [3H]CDP-diacylglycerol. From this indirect evidence and from the results shown in Figs. 2 and 4, it seems that the major event that stimulates the incorporation of [3H]CTP into lipids is an increased activity of phosphatide cytidyltransferase.

Discussion

Phosphatide cytidyltransferase is inhibited by relatively high concentrations of amphiphilic cations in a non-competitive manner (Table 1; Fig. 1). In contrast, phosphatide phosphohydrolase is more sensitive to inhibition by these compounds and the inhibition is competitive with respect to phosphatide (M. Bowley, J. Cooling, S. Burditt & D. N. Brindley, unpublished work). This means that at concentrations of phosphatide as low as those that occur in the cell the inhibition of the phosphohydrolase should be accentuated. Norfenfluramine (Fig. 2a) and chlorpromazine (not shown) inhibited the utilization of membrane-bound [^{14}C]phosphatide by decreasing its conversion into diacylglycerol while at the same time stimulating its flux to CDP-diacylglycerol. These results demonstrate how

cationic drugs enhance the accumulation of phosphatide and how they redirect phospholipid metabolism towards the synthesis of acidic lipids, as was predicted earlier (Brindley *et al.*, 1975).

The increased synthesis of CDP-diacylglycerol was not caused by a greater availability of phosphatide which resulted from the inhibition of the phosphohydrolase (Fig. 2). The addition of partially purified phosphatide phosphohydrolase to the membrane-bound phosphatide enhanced the synthesis of diacylglycerol, but produced no significant change in the rate of CDP-diacylglycerol synthesis. Chlorpromazine and norfenfluramine actively stimulated the cytidyltransferase activity, but only at low concentrations of Mg^{2+} (Fig. 5). The observed interaction of the cationic drugs with phosphatide (Mulé, 1969; Ohki, 1970) will alter the physical state of the phosphatide, including such properties as the effective electrical potential (Papahadjopoulos, 1970). This appears to favour the activity of the cytidyltransferase and inhibits phosphatide phosphohydrolase (Fig. 2a). Higher concentrations of Mg^{2+} have the same effect (Fig. 2b) and remove the stimulation by chlorpromazine and norfenfluramine (Fig. 5). The amphiphilic drugs and Mg^{2+} therefore appear to be having a complementary effect rather than to be acting antagonistically. However, the cationic drugs cannot completely replace the Mg^{2+} , since below the threshold value for Mg^{2+} (Fig. 4) the stimulatory effect of the drugs is not observed. This probably means that the cytidyltransferase recognizes Mg^{2+} phosphatide as its substrate but in addition requires that the phosphatide should be in a suitable physical form. This property and the ability of a bivalent cation to modify the relative flux of phosphatide to diacylglycerol and to CDP-diacylglycerol might be used by the cell to regulate the synthesis of the neutral and acidic glycerolipids. The average concentration of Mg^{2+} in rat liver is about 8 mM (Long, 1961) and thus the effects of Mg^{2+} on phosphatide utilization, which are reported here, occur within the physiological range.

Although the results presented in this paper do provide an explanation for many of the alterations in lipid metabolism that are observed with amphiphilic cations, there may well be other effects. These compounds preferentially interact with acidic phospholipids (Papahadjopoulos *et al.*, 1975) and therefore any enzymes acting on this type of substrate are potential target sites. Propranolol has been shown to increase the synthesis of phosphatidylserine by stimulating the Ca^{2+} -catalysed base-exchange reaction (Abdel-Latif & Smith, 1976). Chlorpromazine and tetracaine inhibit the synthesis of phosphatidyl-inositol from CDP-diacylglycerol and decrease its rate of catabolism (Jungalwala *et al.*, 1971; Freinkel *et al.*, 1975). The net effect of amphiphilic cations

on the metabolism of acidic phospholipids probably depends on which enzyme reaction is rate-limiting, and this probably varies in different tissues and under different physiological conditions.

The general ability of amphiphilic cationic drugs to interact with acidic phospholipids, thus changing their physical properties and also their metabolic turnover, seems to be involved in a number of important pharmacological phenomena. (1) It may partly explain the effects of the fenfluramine derivatives in decreasing the rate of triacylglycerol synthesis and the secretion of very-low-density lipoproteins (Brindley & Bowley, 1975*b*; Brindley *et al.*, 1976). (2) It is probably the basis for the phospholipidosis which is observed after prolonged treatment with high doses of certain amphiphilic cationic drugs (Michell *et al.*, 1976). (3) It is probably involved in the interaction of the drugs with biological membranes and their effects on membrane movement, fusion, permeability, transport and receptor function (Papahadjopoulos *et al.*, 1975; Brindley *et al.*, 1975).

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References

- Abdel-Latif, A. A. & Smith, J. P. (1976) *Biochem. Pharmacol.* **25**, 1697–1704
- Agranoff, B. W. & Suomi, W. D. (1963) *Biochem. Prep.* **10**, 47–51
- Allan, D. & Michell, R. H. (1975) *Biochem. J.* **148**, 471–478
- Brindley, D. N. & Bowley, M. (1975*a*) *Biochem. J.* **148**, 461–469
- Brindley, D. N. & Bowley, M. (1975*b*) *Postgrad. Med. J.* **51**, Suppl. 1, 91–95
- Brindley, D. N. & White, D. A. (1974) *Biochem. Soc. Trans.* **2**, 44–46
- Brindley, D. N., Allan, D. & Michell, R. H. (1975) *J. Pharm. Pharmacol.* **27**, 462–464
- Brindley, D. N., Bowley, M., Burditt, S., Pritchard, P. H., Lloyd-Davies, K. A. & Boucrot, P. (1976) *J. Pharm. Pharmacol.* **28**, 676–682
- Carter, J. R. & Kennedy, E. P. (1966) *J. Lipid Res.* **7**, 678–683
- Davidson, F. M. & Long, C. (1958) *Biochem. J.* **69**, 458–459
- Dawson, R. M. C. & Hauser, H. (1970) in *Calcium and Cellular Function* (Cuthbert, A. W., ed.), pp. 17–41, Macmillan, London
- Eichberg, J. & Hauser, G. (1974) *Biochem. Biophys. Res. Commun.* **60**, 1460–1467
- Eichberg, J. & Hauser, G. (1975) *J. Biol. Chem.* **250**, 105–112
- Freinkel, N., Younsi, C. E. & Dawson, R. M. C. (1975) *Eur. J. Biochem.* **59**, 245–252
- Hajra, A. K., Seguin, E. B. & Agranoff, B. W. (1968) *J. Biol. Chem.* **243**, 1609–1616
- Holub, B. J. & Piekarski, J. (1976) *Lipids* **11**, 251–257
- Hosaka, K., Yamashita, S. & Numa, S. (1975) *J. Biochem. (Tokyo)* **77**, 501–509
- Hübscher, G., West, G. R. & Brindley, D. N. (1965) *Biochem. J.* **97**, 629–642
- Jungalwala, F. B., Freinkel, N. & Dawson, R. M. C. (1971) *Biochem. J.* **123**, 19–33
- Long, C. (1961) in *Biochemists' Handbook*, p. 780, Spon Ltd., London
- Mangiapane, E. H., Lloyd-Davies, K. A. & Brindley, D. N. (1973) *Biochem. J.* **134**, 103–112
- Michell, R. H., Allan, D., Bowley, M. & Brindley, D. N. (1976) *J. Pharm. Pharmacol.* **28**, 331–332
- Mitchell, M. P., Brindley, D. N. & Hübscher, G. (1971) *Eur. J. Biochem.* **18**, 214–220
- Mulé, S. J. (1969) *Biochem. Pharmacol.* **18**, 339–346
- Ohki, S. (1970) *Biochim. Biophys. Acta* **219**, 18–27
- Papahadjopoulos, D. (1970) *Biochim. Biophys. Acta* **211**, 467–477
- Papahadjopoulos, D., Jacobson, K., Poste, G. & Shepherd, G. (1975) *Biochim. Biophys. Acta* **394**, 504–519
- Raetz, C. R. H., Dowham, W. & Kennedy, E. P. (1976) *J. Bacteriol.* **125**, 855–863
- Renkonen, O. (1969) *Biochim. Biophys. Acta* **152**, 114–135
- Sánchez, M., Nicholls, D. G. & Brindley, D. N. (1973) *Biochem. J.* **132**, 697–706
- Singleton, W. S., Gray, M. S., Brown, M. L. & White, J. L. (1965) *J. Am. Oil Chem. Soc.* **42**, 53–56