The Effects of Amphiphilic Cationic Drugs and Inorganic Cations on the Activity of Phosphatidate Phosphohydrolase

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1. Phosphatidate phosphohydrolase from the particle-free supernatant of rat liver was assayed by using emulsions of phosphatidate as substrate. 2. The inhibition of the phosphohydrolase by chlorpromazine was of a competitive type with respect to phosphatidate. The potency of various amphiphilic cationic drugs as inhibitors of this reaction was related to their partition coefficients into a phosphatidate emulsion. 3. The effect of chlorpromazine on the phosphohydrolase activity was complementary rather than antagonistic towards Mg^{2+} . Chlorpromazine stimulated the phosphohydrolase activity in the absence of added Mg^{2+} and was able to replace the requirement for Mg^{2+} . However, at optimum concentrations of Mg^{2+} , chlorpromazine inhibited the reaction, as did Ca^{2+} . The phosphohydrolase activity was also stimulated by Co^{2+} and to a lesser extent by Mn^{2+} , Fe^{2+} , Fe^{3+} , Ca^{2+} , spermine and spermidine when Mg^{2+} was not added to the assays. 4. It is concluded that the inhibition of phosphatidate phosphohydrolase by amphiphilic cations can largely be explained by the interaction of these compounds with phosphatidate, which changes the physical properties of the lipid, making it less available for conversion into diacylglycerol. 5. The implications of these results to the effects of amphiphilic cations in redirecting glycerolipid synthesis at the level of phosphatidate are discussed.

A large variety of amphiphilic cationic drugs, which are used widely in clinical medicine, are able *in vitro* to inhibit phosphatidate phosphohydrolase (EC 3.1.3.4) (Brindley & Bowley, 1975) and to stimulate phosphatidate cytidyltransferase (EC 2.7.7.41) (Sturton & Brindley, 1977). These events may lead to a redirection of glycerolipid metabolism away from the production of triacylglycerol, phosphatidylcholine and phosphatidylethanolamine and towards the synthesis of the acidic phospholipids (Eichberg & Hauser, 1974; Allan & Michell, 1975; Brindley & Bowley, 1975; Abdel-Latif & Smith, 1976; Bazán *et al.*, 1976). Such an action could contribute to the pharmacological effects of some of these drugs (Brindley & Bowley, 1975; Brindley *et al.*, 1975, 1976; Pritchard & Brindley, 1977) and to some of their toxic effects (Michell *et al.*, 1976; Brindley *et al.*, 1977). The ability of these cationic drugs to inhibit phosphatidate phosphohydrolase could be an important property, since this enzyme shows many of the characteristics of a regulatory enzyme (Vavrečka *et al.*, 1969; Mangiapane *et al.*, 1973; Lamb & Fallon, 1974; Fallon *et al.*, 1977).

It was previously proposed that the inhibition of phosphatidate phosphohydrolase by the cationic drugs could be caused by their physical interaction

with phosphatidate (Brindley & Bowley, 1975; Allan & Michell, 1975). This could lead to the displacement of bivalent cations from the phosphatidate and to a change in the charge characteristics around this lipid (Bangham *et al.*, 1965; Papahadjopoulos, 1970; Dawson & Hauser, 1970; Traynor & Kunze, 1975; Lee, 1976). The association of the drugs with phosphatidate is one example of a general interaction with acidic phospholipids.

The present work was therefore undertaken to investigate whether the inhibition of phosphatidate phosphohydrolase could be explained by the proposed model and also to determine the relation between bivalent and amphiphilic cations. Since a large portion of the phosphatidate phosphohydrolase which is active in glycerolipid synthesis in rat liver is isolated in the particle-free supernatant (Hübscher *et al.*, 1967), this fraction was used as a source of enzyme.

Materials and Methods

Male Wistar rats and most of the chemicals used in this investigation were obtained as described previously (Sánchez *et al.*, 1973; Brindley & Bowley, 1975). Spermidine trihydrochloride and spermine

tetrahydrochloride were from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Chlorpromazine hydrochloride [Largactil; 2-chloro-10-(3-dimethylaminopropyl)phenothiazine hydrochloride] was a gift from May and Baker, Dagenham, Essex, U.K., trifluoperazine dihydrochloride {Stelazine; 10-[3-(4-methylpiperazin-1-yl)propyl]-2-trifluoromethylphenothiazine dihydrochloride} was a gift from Smith, Kline and French Laboratories, Welwyn Garden City, Herts., U.K., and amitriptyline hydrochloride {3-(10,11-dihydro-5H-dibenzo[*a,d*]cyclohepten-5-ylidene)-1-(*NN*-dimethylamino)propane hydrochloride} was from Merck, Sharp and Dohme, Hoddesdon, Herts. HN11 9BU, U.K. All solutions were prepared in deionized water and the solutions of chlorpromazine and trifluoperazine were used immediately. The preparation of potassium phosphatide was that described by Sturton & Brindley (1977), except that the phosphatide was converted into its acid form by using the extraction procedure of Hajra *et al.* (1968) immediately before Chelex treatment.

Preparation of the particle-free supernatant from rat liver

This was prepared as described by Sturton & Brindley (1977). The particle-free supernatant was dialysed against 0.3M-sucrose, adjusted to pH 7.4 with KHCO_3 , and containing 0.5mM-dithiothreitol. In the experiments described in Fig. 4, 2.5mM-EDTA, adjusted to pH 7 with KOH, was added to the dialysis medium.

Determination of protein

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

Determination of phosphate

Total phosphate and P_i concentrations were determined by the methods of Bartlett (1959) and Ames (1966) respectively. Amphiphilic amines are known to interfere with the phosphate determination, and these were removed by chloroform extraction before the analysis for P_i (Ueda & Wada, 1970).

Determination of magnesium

Samples were digested at approx. 22°C for 60h after adding 0.1 vol. of conc. HNO_3 . Any precipitates which were formed were removed by sedimentation in a bench centrifuge. The total Mg^{2+} concentration was determined by using a Unicam SP.90 atomic-absorption spectrophotometer and a standard MgCl_2 solution (BDH Chemicals, Poole, Dorset, U.K.), which was treated in the same way as the samples. It was then possible to calculate the concentration of free Mg^{2+} in the assays which contained EDTA by using the calculations described by Raaflaub (1966).

Determination of amphiphilic amines

The concentrations of amphiphilic amines in the experiments performed to measure their partition coefficients were determined by absorption spectroscopy and compared with standard solutions. Absorption measurements were performed at the following wavelengths (extinction coefficients in units of $\text{litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ in parentheses): chlorpromazine at 305nm (3900); cinchocaine at 326nm (3300); mepyramine at 307nm (4800); trifluoperazine at 307nm (4300); desipramine at 252nm (6800); amitriptyline at 250nm (9500); *N*-(2-benzoyloxyethyl)norfenfluramine methanesulphonate (compound S-1513) at 271nm (1400).

Measurement of phosphatide phosphohydrolase activity

The method was similar to those described previously by Sedgwick & Hübscher (1967) and Lamb & Fallon (1974). Unless stated otherwise, each assay contained in a final volume of 0.25ml: 20mM-Tris/maleate buffer, pH 6.4, 1mM-dithiothreitol, 2mg of fatty acid-poor bovine serum albumin/ml, 0.2mM-EGTA, 2mM- MgCl_2 and 1.5mM-potassium phosphatide. The reaction was started by adding 350–400 μg of protein from the particle-free supernatant and stopped after 15 or 20min at 37°C with 0.25ml of 0.61M-trichloroacetic acid. The mixture was cooled on ice for 20min and the precipitate was removed by sedimentation in a bench centrifuge. The quantity of P_i released during the assay was then measured in the supernatant. The amount of P_i recovered after an incubation from which phosphatide was omitted was subtracted as a blank. The reaction rate was constant for at least 25min and proportional to the quantity of particle-free supernatant protein up to a concentration of 3.2mg/ml.

Measurement of the partition coefficients of some amphiphilic cationic drugs

The partitioning of the amphiphilic cationic drugs into an emulsion of phosphatide was measured by a method based on that of Papahadjopoulos *et al.* (1975). An emulsion of potassium phosphatide (0.5mM) was prepared in deionized water by sonicating at 21 kHz for 1–2min under an atmosphere of N_2 . MgCl_2 was added to give a final concentration of 10mM. This mixture was then sonicated for a further 1min at 21 kHz and at about 22°C. The resulting emulsion was then quickly pipetted into an incubation medium, final volume 2ml, containing: 0.16mM-phosphatide, 3.2mM- MgCl_2 , 20mM-Tris/maleate buffer, pH 6.4, 1mM-dithiothreitol, 0.2mM-EGTA and at least five concentrations of the amphiphilic cations. The mixtures were incubated overnight (at least 16h) at 25°C and then centrifuged for 30min at

25°C and 100000g (r_{av} , 7.11 cm). This resulted in the sedimentation of more than 97% of the phosphatidate. The relatively high concentration of Mg^{2+} used in the preparation of the emulsion was essential to ensure the almost complete sedimentation of the phosphatidate. The concentrations of chlorpromazine, cinchocaine, mepyramine and trifluoperazine were measured in the supernatant as described above. However, the strong absorption produced by maleate at 210nm interferes with the determination of compound S-1513, desipramine and amitriptyline. These amines were therefore extracted into chloroform after adjusting the supernatant to pH 12 with 1M-KOH, and their concentration in the organic phase was determined.

The partition coefficient, P , was calculated by using the following formula:

$$P = \frac{[\text{bound cation}]}{[\text{free cation}]} \cdot \frac{\text{wt. of aqueous phase}}{\text{wt. of phosphatidate}}$$

assuming an average mol.wt. of 660 for phosphatidate and a sp.gr. for the aqueous phase of 1.

Experimental and Results

Interaction between phosphatidate and amphiphilic cations and the inhibition of phosphatidate phosphohydrolase activity

Amphiphilic cations are known to interact with phospholipids and to bind to biological membranes (Kawant & Seeman, 1969; Hauser *et al.*, 1969; Papahadjopoulos, 1972; Fernández & Cerbón, 1973; Bickel & Steele, 1974; Scherphof & Westenberg, 1975; Johnson *et al.*, 1976; Wilschut *et al.*, 1976; Seydel & Wasserman, 1976). Hauser *et al.* (1969) and Carey *et al.* (1976) showed that this interaction was specific for acidic phospholipids (e.g. phosphatidylserine) with little interaction with zwitterionic lipids (e.g. phosphatidylcholine). Papahadjopoulos *et al.* (1975) demonstrated that the affinity of interaction between cinchocaine and acidic phospholipids was about 20 times greater than with zwitterionic phospholipids. The purpose of the present experiments was to investigate whether the interaction of the drugs with the phosphatidate substrate could account for the inhibition of phosphatidate phosphohydrolase.

This interaction was demonstrated by measuring the binding of a series of amphiphilic cationic drugs to a phosphatidate emulsion under conditions which resemble those used to assay the activity of phosphatidate phosphohydrolase. The partition coefficients thus obtained cannot be used directly to calculate the interactions of the drugs with phosphatidate in the phosphohydrolase assays. These assays contain different molar proportions of Mg^{2+} and phosphatidate from those used to measure the partition coefficients, and they also included albumin

and the enzyme preparation. However, it is valid to compare the rank order of the partition coefficients for the drugs with the rank order of their inhibitory potentials. The logs of partition coefficients of the drugs into the phosphatidate emulsion were plotted against the log of the reciprocal concentration of drug required to inhibit phosphatidate phosphohydrolase activity by 50%. This mathematical treatment is described by Hansch & Glave (1971). The hydrophobicity of the drugs is directly correlated with the ability to inhibit the phosphohydrolase ($r = 0.96$; $P < 0.001$; Fig. 1).

From these results it was predicted that the inhibition of phosphatidate phosphohydrolase by the drugs should be of a competitive type with respect to phosphatidate. This prediction was tested by using chlorpromazine as an example of an amphiphilic amine (Fig. 2). The lines obtained in a double-reciprocal plot with different concentrations of chlorpromazine appear to intersect in the first quadrant. The intercepts on the $1/v$ axis for the lines obtained at 0.6mM- and 1.0mM-chlorpromazine were -0.45 ± 0.39 (s.d.) and 0.17 ± 0.35 (s.d.) respectively. The intercept for the corresponding line in the absence of chlorpromazine was 1.05 ± 0.09 (s.d.) and

Fig. 1. Relationship between the potency of some amphiphilic cationic drugs in inhibiting phosphatidate phosphohydrolase and their partition coefficients into a phosphatidate emulsion

The assay of phosphatidate phosphohydrolase and the measurement of the partition coefficients are described in the Materials and Methods section. The error bars show the range of the partition coefficients obtained from at least two independent experiments. ED_{50} refers to the concentration of drug required to produce a 50% inhibition of phosphatidate phosphohydrolase activity, and this value is accurate to within 10%. It was calculated from the results of three independent experiments as described by Dodds *et al.* (1976). ○, Mepyramine; □, cinchocaine; △, compound S-1513; ▽, amitriptyline; ●, desipramine; ■, chlorpromazine; ▲, trifluoperazine.

Fig. 2. Inhibition of phosphatidate phosphohydrolase by chlorpromazine

Phosphatidate phosphohydrolase activity was measured as described in the Materials and Methods section except that 5 mM-MgCl₂ was present in the assays. The concentrations of chlorpromazine were: ○, 0 mM; ●, 0.3 mM; △, 0.45 mM; ▲, 0.6 mM; □, 1.0 mM. The results were obtained with six different preparations of phosphohydrolase and are expressed relative to the calculated maximum velocity in the individual experiments. These were calculated by Lineweaver & Burk (1934) analysis by using a weighted regression procedure (Wilkinson, 1961) from the results obtained in the absence of chlorpromazine. The mean value of $V_{\max}$ and 'apparent K_m ' for phosphatidate were 1.4 ± 0.2 (S.E.M.) nmol of phosphatidate hydrolysed/min per mg of soluble protein and 0.42 ± 0.08 (S.E.M.) mM respectively. The error bars on the graph are based on the S.E.M. of the mean relative velocities.

was significantly greater ($P < 0.05$) than these values. Taking into account the S.D. for the slopes and intercepts of all of the lines as calculated by Wilkinson (1961) analysis, the results are consistent with there being a common point of intersection in the range where $1/[\text{phosphatidate}] = 0.5\text{--}0.7 \text{ mM}^{-1}$ (Fig. 2). The results in Fig. 2 are therefore consistent with the type of competitive inhibition that involves the interaction of the inhibitor with one of the substrates of the reaction (Sluiterman & Wijdenes, 1973), and such an interaction was demonstrated in Fig. 1.

However, we cannot calculate accurate values for the association constant of phosphatidate and chlorpromazine and the K_i for chlorpromazine. This is because phosphatidate is not soluble in water at the concentrations used and its effective concentration with respect to phosphatidate phosphohydrolase is not known. The competition between Mg²⁺ and chlorpromazine in binding to phosphatidate will also complicate the calculation of these values.

Effects of inorganic and amphiphilic cations on the activity of phosphatidate phosphohydrolase

Phosphatidate phosphohydrolase activity has been studied in a number of cell types and cell organelles, and there appear to be different phosphohydrolases with different physiological functions (Brindley, 1974). The effects of inorganic cations on these activities have been investigated and the results show apparent conflict, which may partly be produced by there being different enzyme activities. Some differences may also be caused by the method of substrate presentation (i.e. membrane-bound phosphatidate or phosphatidate emulsions), which differs in the various reports. Other problems may result because phosphatidate is normally prepared by treatment of phosphatidylcholine in the presence of phospholipase D (EC 3.1.4.4) and Ca²⁺ ions. These Ca²⁺ ions are tightly bound to phosphatidate and the extent of their removal will influence the effects of other ions on the phosphohydrolase activity.

There are reports that phosphatidate phosphohydrolase activity is not stimulated by bivalent cations (Coleman & Hübscher, 1962; Smith *et al.*, 1967; Caras & Shapiro, 1975; Kent & Vagelos, 1976). By contrast, Mg²⁺ ions have been reported to stimulate phosphohydrolase activity (Sedgwick & Hübscher, 1965, 1967; Mitchell *et al.*, 1971; Jamdar & Fallon, 1973; Call & Williams, 1973; Lamb & Fallon, 1974; Hosaka *et al.*, 1975; Sturton & Brindley, 1977). Other bivalent cations either stimulate (Sedgwick & Hübscher, 1965) or inhibit (Jamdar & Fallon, 1973; Lamb & Fallon, 1974). There also appear to be two different phosphatidate phosphohydrolase activities in liver, heart and adipose tissue: the mitochondrial activities were not activated by Mg²⁺, whereas those in the microsomal and supernatant fractions were (Jamdar & Fallon, 1973; Lamb & Fallon, 1974; Kako & Patterson, 1975).

The effects of a variety of cations on phosphatidate phosphohydrolase activity were investigated under the conditions used in the present experiments (Table 1). The activity was markedly stimulated by Mg²⁺ and to a lesser extent by Co²⁺. Other cations, including Mn²⁺, Fe²⁺, Fe³⁺, Ca²⁺, spermine and spermidine, stimulated it slightly, whereas univalent cations were without effect. The maximum activity of the phosphohydrolase was surprisingly obtained in

Table 1. *Effects of various cations on the activity of phosphatidate phosphohydrolase*

Phosphatidate phosphohydrolase activity was measured as described in the Materials and Methods section except that in the basic assay system EGTA and Mg^{2+} were omitted. The rate of phosphatidate hydrolysis under these conditions was 0.4 ± 0.1 (s.d. from five independent experiments) nmol/min per mg of soluble protein. The rates of reaction in the presence of additional cations are expressed relative to this value. The numbers of independent experiments performed are given in parentheses and the results are expressed as means $\pm$ 1 s.d.

Cation	Concentration range which gives maximum activity (mM)	Relative maximum velocity
None	—	1.00
Chlorpromazine	1–1.75	5.2 ± 1.7 (5)
Mg^{2+}	2–5	4.5 ± 1.3 (5)
Co^{2+}	0.4–0.6	3.0 ± 0.6 (3)
Mn^{2+}	0.15–0.55	1.9 ± 0.4 (3)
Spermidine	0.5–3.0	1.5 ± 0.2 (3)
Fe^{2+}	0.3–0.5	1.5 ± 0.02 (3)
Spermine	0.13–0.5	1.4 ± 0.3 (3)
Fe^{3+}	0.2–0.5	1.3 ± 0.1 (3)
Ca^{2+}	0.25–0.5	1.3 ± 0.2 (3)
K^+	10–400	0.97 ± 0.14 (3)
Na^+	10–400	0.96 ± 0.18 (3)
NH_4^+	10–400	0.98 ± 0.20 (3)

the presence of 1–1.75 mM-chlorpromazine, which was used as an example of an amphiphilic cation. The molar ratio of phosphatidate/chlorpromazine in the assay system which gave this activity was approximately 1:1.

This stimulation by chlorpromazine and its interrelationship with the concentration of Mg^{2+} was studied further (Fig. 3). The maximum stimulation by chlorpromazine was achieved over a narrow range of concentrations, and at pH 6.4 was greater ($P < 0.05$ from five independent experiments) than that obtained with Mg^{2+} . Higher concentrations of chlorpromazine caused the activity to decrease rapidly, and a similar effect was obtained with high concentrations of the bivalent inorganic ions that were investigated. As the concentration of Mg^{2+} was increased in the assay, the relative stimulation by chlorpromazine was decreased ($P < 0.05$ from three independent experiments; see also Fig. 3), and the concentration of the amine required for optimum activity was lowered. At pH 7, the maximum stimulations produced by chlorpromazine and Mg^{2+} were not significantly different (Fig. 4a).

The phosphatidate used in these experiments was freed of bivalent cations by the acid extraction and Chelex chromatography used in its preparation (see the Materials and Methods section). The enzyme preparation used in the assay was also dialysed over-

Fig. 3. *Effects of Mg^{2+} and chlorpromazine on the activity of phosphatidate phosphohydrolase*

The Figure shows the results of one representative experiment. The concentrations of Mg^{2+} in the assays were: Δ , 0 mM; $\circ$, 0.3 mM; $\blacksquare$, 0.6 mM; $\square$, 1 mM; $\bullet$, 1.5 mM. In five independent experiments the activity in the absence of Mg^{2+} and chlorpromazine was 0.38 ± 0.05 (s.e.m.), the V_{max} in the presence of optimum $MgCl_2$ was 1.76 ± 0.07 (s.e.m.) nmol of phosphatidate hydrolysed/min per mg of supernatant protein and the K_m value for $MgCl_2$ was 0.32 ± 0.1 (s.e.m.) mM. The last two values were obtained by weighted regression analysis as described in Fig. 2.

night. Some of the problems encountered in the past (Smith *et al.*, 1967) in using phosphatidate emulsions to assay the soluble phosphohydrolase may have been caused by the Ca^{2+} that was probably still bound to the substrate. In the present experiments, 1.8 mM- $CaCl_2$ caused a 50% inhibition of phosphohydrolase activity in the presence of 5 mM- $MgCl_2$.

Although Fig. 3 appears to show that chlorpromazine can efficiently replace the requirement for Mg^{2+} , it does not exclude the possibility that phosphatidate phosphohydrolase might require low concentrations (in the μM range) of Mg^{2+} for activity. This possibility was investigated in the experiments described in Fig. 4. These assays were performed at pH 7.0 rather than at 6.4 to increase the chelation of Mg^{2+} by EDTA. The total concentration of Mg^{2+} in the basic assay system in three independent experiments was 9–13 μM and was derived mainly from the

Fig. 4. Effects of chlorpromazine, Mg^{2+} and EDTA on the activity of phosphatidate phosphohydrolase at pH 7. The particle-free supernatant used in these experiments was dialysed against 0.3M-sucrose containing 0.5mM-dithiothreitol and 2.5mM-EDTA, adjusted to pH7 with KOH. The pH of the assay was increased from pH6.4 to 7.0 to increase the chelation of Mg^{2+} to the EDTA. The basic assay system contained in a volume of 0.25 ml: 20mM-Tris/maleate buffer, pH7, 1mM-dithiothreitol, 1.5mM-potassium phosphatidate, 0.5mg of fatty acid-poor bovine serum albumin and 0.4mg of particle-free supernatant protein. This basic assay medium also contained 0.3–0.4mM-EDTA, which was derived from the enzyme preparation. The phosphohydrolase activity was measured over 20 min at 37°C (see the Materials and Methods section). (a) Effects of adding EDTA to the basic system (●) or to incubations which contained either 2mM- $MgCl_2$ (▲) or 1.2mM-chlorpromazine (■). The rates are expressed relative to the activity obtained in the absence of EDTA, Mg^{2+} and chlorpromazine. The error bars when indicated show the s.d. from three independent experiments and otherwise show the range from two independent experiments. (b) Effect of changing the concentration

enzyme preparation. The concentration of 'free' Mg^{2+} was in the range 56–76pM, since the system contained 0.3–0.4mM-EDTA from the enzyme preparation. Further addition of EDTA stimulated the basal activity by about 1.5-fold (Fig. 4a). When 2mM- $MgCl_2$ was added to the basic system the concentration of 'free' Mg^{2+} was calculated to be 1.6–1.8mM, and this produced an optimum stimulation of phosphohydrolase activity. The further addition of 8mM-EDTA decreased the concentration of 'free' Mg^{2+} to 0.6–0.7nM and the phosphohydrolase activity under these conditions was the same as if no Mg^{2+} had been added.

By contrast, EDTA failed to inhibit completely the chlorpromazine-stimulated activity (Fig. 4). An optimum stimulation of phosphohydrolase activity was obtained with 1.2mM-chlorpromazine (Fig. 4b). The total concentration of Mg^{2+} in these assays was 12–16 μ M, whereas the calculated concentration of 'free' Mg^{2+} in three independent experiments was 80–100pM. The further addition of 8mM-EDTA decreased the concentration of 'free' Mg^{2+} to 3–4pM, but the phosphohydrolase activity was still $92 \pm 5\%$ (s.d. from three independent experiments) of its original value. Higher concentrations of EDTA could further inhibit the phosphohydrolase activity (Figs. 4a and 4b), but even at 24mM-EDTA this inhibition reached only 31–41% (from two independent experiments). In these assays the concentration of 'free' Mg^{2+} was approx. 1pM. It is likely that this high concentration of EDTA inhibited the phosphohydrolase directly rather than by complexing Mg^{2+} .

Discussion

The results in the present paper are compatible with the following explanation for the inhibition of phosphatidate phosphohydrolase by amphiphilic cations. The drugs interact with the phosphatidate (Figs. 1 and 2) and two parts of their structure are involved. The hydrophobic region of the drug partitions into the biological or artificial membrane in which phosphatidate is present. This explains the correlation between the inhibitory potential of the drug and its partition coefficient (Fig. 1). In addition, the positively charged amine group is attracted by electrostatic forces to the negatively charged phos-

of chlorpromazine in assays which contained no further addition of EDTA (○), an additional 8mM-EDTA (□) and an additional 16mM-EDTA (Δ). The error bars, where shown, represent the s.d. from three independent experiments, except on the 8mM-EDTA curve, where they are the ranges from two independent experiments.

phate group (Hauser *et al.*, 1969; Cerbón, 1972; Papahadjopoulos, 1972; Giotta *et al.*, 1974; Ragazzi *et al.*, 1975; Carey *et al.*, 1976; Lee, 1976). This explains why carboxymethylnorfenfluramine [2-(carboxymethylamino)-1-(3'-trifluoromethylphenyl)propane], which has an anionic group next to the amine, is not an inhibitor of phosphatidate phosphohydrolase (Brindley *et al.*, 1977). Sotalol [4'-[1-hydroxy-2-(isopropylamino)ethyl]methanesulphonanilide] (Abdel-Latif & Smith, 1976) and 2-[(3'-chloro-4'-sulphamoylbenzoyloxy)ethylamino]-1-(3"-trifluoromethylphenyl)propane (compound S-1204) (Brindley & Bowley, 1975), which have a polar group in the hydrophobic region, are also poor inhibitors of phosphatidate phosphohydrolase. By contrast, amphiphilic anions such as 4-(4'-chlorophenyl)-phenoxyisobutyrate, 2-(4'-chloromethyl)-2-(3'-trifluoromethylphenoxyl)acetate and 4-chlorophenoxyisobutyrate stimulate the activity of the phosphohydrolase at concentrations where the amphiphilic cations inhibit (Brindley & Bowley, 1975).

The inhibition of phosphatidate phosphohydrolase by amphiphilic amines probably results from a change in the physical properties of the phosphatidate. These could include the packing arrangement of the lipid in the membrane and its effective electrical potential (Bangham *et al.*, 1965; Papahadjopoulos, 1970; Papahadjopoulos *et al.*, 1975; Lee, 1976; Wilschut *et al.*, 1976). As the phosphatidate becomes more positively charged the phosphohydrolase activity is first favoured and then inhibited.

The results in Figs. 3 and 4 show that, although Mg^{2+} enhanced the phosphohydrolase activity more than did other inorganic cations, it cannot be considered a cofactor in the sense that an Mg^{2+} -phosphate linkage is specifically required for enzyme activity. This is demonstrated by the fact that chlorpromazine can substitute for Mg^{2+} (Table 1, Figs. 3 and 4) and that the chlorpromazine-stimulated activity is relatively insensitive to inhibition by EDTA (Fig. 4). The requirement for Mg^{2+} could be partially replaced by Co^{2+} and Mn^{2+} . However, Ca^{2+} was much less effective. Although Ca^{2+} may change the physical properties of the phosphatidate emulsion in a similar way to Mg^{2+} , it also seems to be an inhibitor of phosphatidate phosphohydrolase activity.

These results have implications in defining specific assay conditions for different phosphohydrolases such as the Mg^{2+} -dependent and Mg^{2+} -independent activities described by Jamdar & Fallon (1973), Lamb & Fallon (1974) and Kako & Patterson (1975). It was hoped in our laboratory that assays in the presence of Mg^{2+} and EDTA respectively would specifically measure these activities, but the results in Figs. 3 and 4 indicate that this is not so. It is, however, possible that different phosphatidate phosphohydrolase activities could be defined in terms of the

physical properties of the phosphatidate substrate with which they are able to react.

Phosphatidate and its complex with amphiphilic cationic drugs are also a potential substrate for phosphatidate cytidyltransferase. On the whole, the effects of amphiphilic cations on the latter activity were different from those observed with the phosphohydrolase. In both enzyme reactions Mg^{2+} and amphiphilic cations were complementary rather than antagonistic in their effects (Table 1; Fig. 3; Sturton & Brindley, 1977). However, the cationic drugs could not completely replace the requirement for Mg^{2+} in the cytidyltransferase reaction. Interaction with relatively high concentrations of amphiphilic cations favours the formation of CDP-diacylglycerol, whereas it inhibits diacylglycerol synthesis (Sturton & Brindley, 1977). The inhibition by high concentrations of cationic drugs in the presence of Mg^{2+} is of a competitive type with respect to phosphatidate for the phosphatidate phosphohydrolase (Fig. 2), but is of a non-competitive type in the cytidyltransferase reaction (Sturton & Brindley, 1977). Since the inhibition is competitive for the phosphohydrolase reaction, the concentration of drug required to produce a 50% inhibition (Fig. 1; Brindley & Bowley, 1975) only describes the rank order of the potencies of the drugs. The phosphatidate in the cell would be expected to interact with bivalent cations such as Mg^{2+} and this should favour the inhibition by the drugs.

Amphiphilic amines may also interact directly with phosphatidate phosphohydrolase and thus change its activity. However, the interaction of amphiphilic cationic drugs with phosphatidate itself provides a reasonable explanation for the simultaneous inhibition of phosphatidate phosphohydrolase and the stimulation of phosphatidate cytidyltransferase.

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