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Kinetic and topographical studies of the phosphatidylcholine : ceramide choline phosphotransferase in plasma membrane particles from mouse ascites cells

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The content of endogenous phospholipids in plasma membrane preparations from Ehrlich ascites cells was depleted by exposure to phospholipase C. The enzyme catalyzing the phosphatidylcholine : ceramide choline phosphotransferase reaction was inactivated by this treatment. However, the activity could be restored with exogenous phosphatidylcholines, demonstrating the dependence of the reaction upon the presence of this substrate. Phosphatidylcholines containing unsaturated fatty acids were 10-fold more effective substrates than the saturated molecular species. The activation energy of the reaction was determined to be 17.2 kcal / mol. Selective trypsin treatment of the plasma membranes suggests that the cholinephosphotransferase may have an asymmetric orientation. The reaction kinetics followed a rate equation similar to that of the ping-pong reaction mechanism, which suggests the formation of an enzyme-bound intermediate of the phosphocholine group being transferred. These results are discussed in terms of possible biological functions of the enzyme.

Introduction

Sphingomyelin is produced by the transfer of the phosphocholine group from phosphatidylcholine directly to ceramide and this reaction has been shown to occur in mouse fibroblasts [1]. This phosphatidylcholine : ceramide cholinephosphotransferase pathway has been established as the principal pathway for sphingomyelin formation in these cells. The possible role of CDPcholine as the immediate donor of the phosphocholine group was eliminated through the direct comparison of the specific radioactivities of CDPcholine to sphingomyelin in the course of [^{14}C]choline incorporation into the cellular phospholipids of mouse fibroblast cultures [2].

These results were confirmed by investigations with other types of mammalian cell [3–5]. This cholinephosphotransferase activity was characterized as a plasma-membrane-bound enzyme [6,4,13] with considerable specificity for ceramides and diacylglycerols [7], the natural acceptor molecules for the phosphocholine group being transferred. There is a possibility that some activity may be associated with the Golgi [8,14].

The cholinephosphotransferase reaction preferentially utilized the endogenous lipid substrates of the plasma membranes. The total activity determined in membrane fractions derived from the fibroblast cultures was found sufficient to account for the observed de novo sphingomyelin synthesis in these cells [8].

Under in vitro conditions, the rate of

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sphingomyelin formation was shown to depend upon the limited supply of free ceramide available in the membrane containing the cholinephosphotransferase activity [8]. It was concluded that the ratio of ceramide and diacylglycerol in the plasma membrane is an important factor controlling the enzyme in vivo [7].

Interest in this cholinephosphotransferase reaction relates to the potential role of the enzyme in the process of plasma membrane formation and turnover.

These considerations relate to the topography, to the regulation and to the reaction mechanism of the enzyme catalyzing the cholinephosphotransferase reaction. In this work, selective trypsin treatment and membrane perturbation by ultrasound is utilized to study topographical aspects of the reaction. Phospholipase-induced delipidation of that membrane and controlled relipidation with individual phospholipids is used to characterize the substrate specificity of the enzyme with respect to the type of phospholipid and the degree of unsaturation in the fatty acyl moiety of phosphatidylcholine.

The dependence of the reaction velocity on temperature as well as the effects of varying concentrations of phospholipid substrates were studied in attempts to identify the characteristics of the particle-bound enzyme.

Materials and Methods

Chemicals. Phosphatidyl[^{14}C]choline (with a specific activity of 10.3 Ci/mol) and [^{3}H]ceramide were prepared as previously described [6,7]. Thin-layer chromatography plates (Silica gel G) were from Merck, Darmstadt, F.R.G.; all other chemicals were obtained from Sigma Chemicals, Munich, F.R.G. Phospholipase A_2 (lot number 507-8040) was from *Naja naja* and had 970 units/mg protein; phospholipase C was from either *Clostridium perfringens* (lot number 59C-6880) having 210 units/mg protein or from *Bacillus cereus* (lot number 1170-0216) and had 120 units/mg protein; Phospholipase D (lot number P-7758) was from cabbage and had 105 units/mg protein. Phosphatidylserine (bovine brain) sphingomyelin (bovine brain), phosphatidylethanolamine (soybean) and phosphatidylcholines (soybean, bovine brain), ceramides (bovine brain), dipalmitoylphosphatidylcholine and distearoylphosphatidylcholine were purchased from Sigma Chemical. Diarachidonoyl-, dilinoleoyl- and dioleoylphosphatidylcholine were purchased from Serva Fine Chemicals, Heidelberg, F.R.G.

Enzyme preparations. Plasma membranes from log-phase mouse Ehrlich ascites cells were prepared according to the method previously described [7].

Incubations. The phosphatidylcholine : ceramide cholinephosphotransferase activity was determined by measuring the quantity of [^{14}]sphingomyelin produced from the phosphatidyl[^{14}C]choline substrate [7]. The incubation mixtures contained 7.2 nmol phosphatidyl[^{14}C]choline (0.057 μCi), 1.2 mg defatted serum albumin, 180 nmol $MnCl_2$, 600 nmol imidazole buffer (pH 6.5) and the enzyme source in a total volume of 60 μl. The incubations were for 6 h at 38°C.

Determination of radioactive sphingomyelin. A combination of the methods of Folch et al. [15] and Dittmer and Wells [16] was used for the isolation of the sphingomyelin formed by cholinephosphotransferase. 1 ml of 0.2 M methanolic NaOH was added to the incubation mixture to terminate the reaction and the turbid suspension was incubated for 30 min at 55°C in order to cleave the ester lipids. The tubes were cooled to 20°C and 2 ml chloroform containing 12 μg/ml of sphingomyelin carrier were added, immediately followed by 0.5 ml of 0.45 M HCl. A two-phase system formed with a film of protein separating the phases. The upper phase and the protein were removed by aspiration and the lower phase was washed with 2 ml of methanol/water (1 : 1, v/v). The upper phase was removed and the lower phase containing the sphingomyelin was taken to dryness with a stream of nitrogen. The residue was dissolved in 50 μl of chloroform and chromatographed on silica gel with $CHCl_3/CH_3OH/HCOOH/H_2O$ (100 : 50 : 12 : 5) as solvent. Identification and quantitation of the radioactive sphingomyelin was performed as described previously [6]. The method was especially useful when phosphatidyl[^{14}C]choline was the radioactive substrate due to the ease of removal of glycerophosphorylcholine produced upon saponification of unreacted PC with the upper phase and the results

were reproducible within 2% of the mean.

In some experiments [^{3}H]ceramide was used as substrate for the determination of sphingomyelin synthesis. 20 nmol (0.35 μCi) of the [^{3}H]ceramide were included in the incubation as a mixed sonicate composed of 1 mg bovine brain sphingomyelin, 0.5 mg lysophosphatidylcholine and 1 mg carrier ceramide prepared from bovine brain sphingomyelin. In some experiments, diacylglycerols were included in the incubation mixture as a sonicate with sphingomyelin and lysophosphatidylcholine using the same procedure as for ceramide.

Phospholipase treatment. Three types of phospholipase were used separately at various concentrations for the phospholipid depletion of plasma membranes in an incubation mixture containing 150 μl of a 7 mg protein/ml aqueous plasma membrane suspension, 150 μl of 250 mg/ml defatted bovine serum albumin, 250 μl of 120 mM Tris (pH 7.5), 70 μl of 10 mM $CaCl_2$ and 150 μl of the aqueous solution of the respective phospholipase at the several concentrations indicated. Phospholipase A_2 from *Naja naja* was present at 30, 5, 0.8 and 0.14 units/ml, phospholipase C from *B. cereus* was present at 30, 5, 0.8 and 0.18 units/ml, phospholipase C from *C. perfringens* was present at 30, 3.7 and 0.24 units/ml. The incubations were stopped after 35 min at 22°C by the addition of 20 ml 0.1 mM EDTA (pH 7.0) and the soluble phospholipases were removed by centrifuging at $10^5 \times g$ for 90 min. The sediment representing the membranes with modified lipid content was washed two times with 10% sucrose and resuspended in water.

Phospholipase D treatment required modified conditions for incubation due to the acidic pH optimum for the enzyme activity and was carried out as follows: 6 mg of the plasma membrane protein were incubated with 150, 7.5 and 0.4 units of cabbage phospholipase D in a volume of 2.5 ml 50 mM sodium acetate buffer (pH 5.6) containing 25 mM $CaCl_2$. The incubation continued for 1 h at 25°C and the reaction was stopped with 20 ml of 10 mM EDTA (pH 7.0) and the phospholipase D was removed by centrifugation as described above.

The presence of residual phospholipase activity was measured in all samples with phosphatidyl[^{14}C]choline under the assay conditions for cholinephosphotransferase activity. There was never more than 3.1% of the substrate cleaved in any of the samples. In addition, the ability of phospholipase C to catalyze the formation of sphingomyelin was independently estimated. No activity was found under the conditions for the cholinephosphotransferase assays. The total phospholipid content was also estimated on the phospholipase-treated samples and the values were compared to that of controls incubated in the absence of phospholipase.

Trypsin treatment of whole cells. 5 ml of packed Ehrlich ascites cells were suspended in 50 ml of 15 mM potassium phosphate buffer (pH 7.6) containing 1% NaCl (w/w) and 2 mM $CaCl_2$ at 20°C. To 10 ml aliquots of the suspension were added 20, 4 and 0.8 mg of trypsin (12000 BAEE units/mg) and the incubation was continued for 15 min at 20°C. Coagulated material which occasionally formed at 2.0 mg/ml trypsin was removed and the tryptic hydrolysis was terminated by the addition of a solution containing 40 mg soybean trypsin inhibitor with a capacity to inhibit 18000 BAEE units of trypsin/mg. The cell suspensions were centrifuged at $200 \times g$ and washed twice with the same buffer containing 1 mg/ml trypsin inhibitor. Trypsin was omitted from the otherwise identically treated control sample.

The plasma membrane fraction was prepared from these cells following the same method as given previously [8] with the exception that the homogenization buffer contained 1 mg/ml trypsin inhibitor.

Trypsin treatment of plasma membranes. To a 1 ml aliquot containing 6 mg protein of plasma membrane were added 0.4 ml of 100 mM Tris buffer (pH 7.6) and 100 μl of a 30, 6 or 1.2 mg/ml trypsin solution. The samples were incubated for 15 min at 20°C and the reaction was stopped by the addition of 100 μl of a solution containing 40 mg soybean trypsin inhibitor/ml. The plasma membrane fraction was washed with a 0.1 mg/ml solution of trypsin inhibitor and resuspended in water.

Sonic treatment of plasma membranes. A sample containing 4 ml of a 2 mg/ml aqueous suspension of plasma membrane particles was sonicated with the microtip of a Branson sonifier at 0°C (ice

cooling) set at position 1.5 corresponding to 20 W. Aliquots were withdrawn after 1, 3, 10 and 30 s and tested directly for cholinephosphotransferase activity in duplicate. These observations were carried out on three separate occasions.

Temperature dependence. The temperature dependence was independently determined with [^{3}H]ceramide or phosphatidyl[^{14}C]choline as the radioactive substrate in the range from 9 to 48°C. The values are the mean of triplicate determinations from four experiments.

Data. If not otherwise stated, the data represent the means of duplicate determinations from two independent experiments. The standard deviation was found to be generally within ± 10% of the mean. In some cases the range of values is given.

Results

The effects of trypsin on the phosphatidylcholine: ceramide cholinephosphotransferase activity

Plasma membrane preparations from Ehrlich ascites cells were subjected to trypsin cleavage prior to incubations for determining cholinephosphotransferase activity. These experiments were undertaken in an attempt to obtain information on the topology of the cholinephosphotransferase molecule on the isolated plasma membrane particles. In a second type of experiment the effects of trypsin were restricted to the outer half of the plasma membrane bilayer by exposing the intact cells to the trypsin prior to the isolation of the plasma membrane fraction and the subsequent determination of the cholinephosphotransferase activity.

In both cases the intact cell or the plasma membrane preparations were exposed to various amounts of trypsin for 15 min at 20°C and the cholinephosphotransferase activity was determined by estimating the formation of radioactive sphingomyelin from either phosphatidyl[^{14}C]-choline or [^{3}H]ceramide.

The results given in Table I indicate that there was little effect on the cholinephosphotransferase activity if the intact whole cell was exposed to trypsin. At the highest concentration of trypsin, the specific activity of the enzyme is 92 ± 5% of that determined with the control plasma membrane fraction.

TABLE I

THE EFFECTS OF TRYPSIN PRETREATMENT OF PLASMA MEMBRANES OR INTACT CELLS ON THE CHOLINEPHOSPHOTRANSFERASE ACTIVITY

Plasma membranes from Ehrlich ascites cells were incubated with trypsin at the indicated concentrations at 20°C for 15 min. Intact Ehrlich ascites cells were subjected to the same treatment and the plasma membrane fraction was isolated. The cholinephosphotransferase activity was determined in both the plasma membrane preparations. The values for the activity are expressed as a comparison to native plasma membranes from untreated cells ($n = 4$).

Sample	[Trypsin] (mg/ml)	Spec. act. cholinephosphotransferase (% of untreated sample)
Whole cells	2.00	92 ± 5
Whole cells	0.40	94 ± 5
Whole cells	0.08	97 ± 5
Plasma membranes	2.00	24 ± 14
Plasma membranes	0.40	41 ± 17
Plasma membranes	0.08	96 ± 31

In contrast, the cholinephosphotransferase activity is decreased in isolated plasma membrane fractions that had been exposed to trypsin. At a concentration of 2.0 mg/ml trypsin, 76 ± 14% of the activity is destroyed, with smaller decreases at the lower trypsin concentrations. The variability of the data reflected in the S.D. is most likely related to the unspecific loss of protein from the membranes. The residual activity of 24 ± 14% of the original value may be associated with a population of closed vesicles which could have formed during the procedure used for the preparation of the plasma membrane fraction. Vesicular structures were indeed visible on electron micrographs of the plasma membrane preparation.

The lack of effect by trypsin treatment of intact cells on cholinephosphotransferase activity of subsequently isolated plasma membrane suggests that cholinephosphotransferase cleavage sites are not exposed on the external surface of these cells.

The effects of sonic perturbation of the plasma membrane structure

Plasma membrane preparations were subject to sonication at 0°C for various time intervals. The effect on sphingomyelin formation was de-

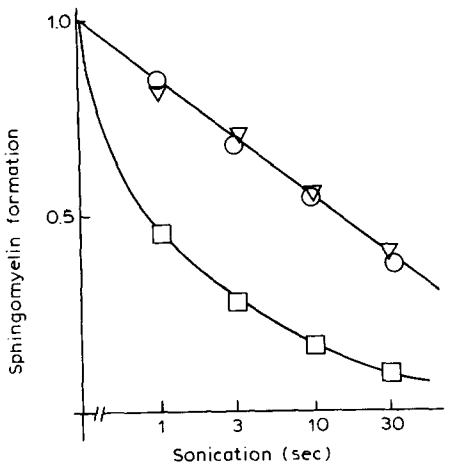

Fig. 1. Effects of sonic perturbation of the plasma membrane. Plasma membrane preparations were subject to sonication with 5 W/ml at 0°C for various time intervals. The cholinephosphotransferase activity was measured as a function of time using three different substrate conditions: ○, phosphatidyl-[^{14}C]choline, exogenous ceramide added; ▽, exogenous [^{3}H]ceramide added, exogenous phosphatidylcholine omitted; □, phosphatidyl[^{14}C]choline added, exogenous ceramide omitted. The results are expressed as the ratio of activity in an untreated sample to that remaining after sonication.

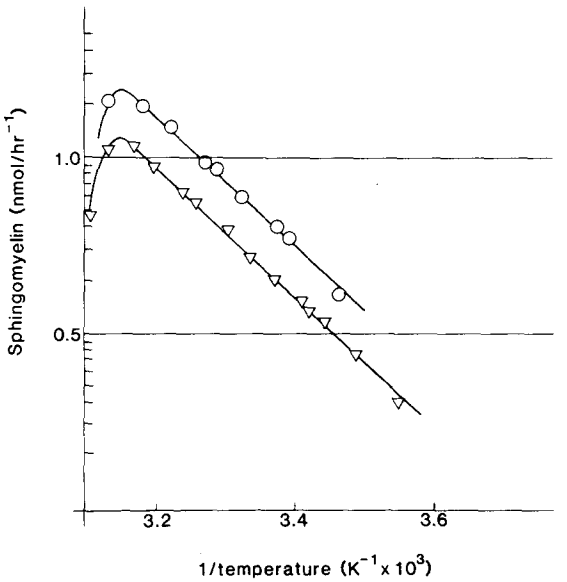

Fig. 2. Temperature dependence of the cholinephosphotransferase reaction. Arrhenius plot of the reaction rates determined with (○) [^{3}H]ceramide and (▽) phosphatidyl[^{14}C]choline. The activity is expressed as nmol/mg protein per h. Activation energy: 17.2 ± 0.2 kcal/mol.

termined using three different types of incubation conditions for the assay of enzyme activity: (1) [^{3}H]ceramide in the absence of exogenous phosphatidylcholine; (2) phosphatidyl[^{14}C]choline in the absence of exogenous ceramide; and (3) phosphatidyl[^{14}C]choline in the presence of exogenous ceramide.

As seen in Fig. 1, this treatment results in a loss of cholinephosphotransferase activity. The three samples exhibited less than 42% of their original enzyme activity after 30 s of sonication. A significantly greater loss of activity to 10% of the control value was observed in the sample which was assayed in the absence of an exogenous supply of the ceramide substrate. This suggests that enzyme denaturation alone is not the sole reason for the loss of activity. Under these conditions the disruption of the topology of the native plasma membrane structure may dissociate the enzyme from its supply of endogenous ceramide, but not from endogenous phosphatidylcholine.

The effects of temperature on the reaction

The temperature dependence of the cholinephosphotransferase reaction was examined to determine if there were any effects on the phase transitions of the exogenous lipid substrates and/or of phase transitions on the lipid microenvironment of the plasma-membrane-bound enzyme. Temperature-induced transitions of this type can be expected to result in abrupt changes of the activation energy of the enzyme-catalyzed reaction.

Sphingomyelin formation was determined separately with phosphatidyl[^{14}C]choline and [^{3}H]-ceramide as the labelled lipid substrate over a range of temperature from 9°C to 48°C. At temperatures below 9°C experimental errors increased due to the low production of labelled sphingomyelin and the partial precipitation of the lipid substrates in the incubation mixture. The Arrhenius plots derived from two independently determined observations for the reaction rate are provided in Fig. 2. Two parallel lines are obtained which are linear between 9°C and 43°C. Heat inactivation of the enzyme starts at temperatures above 44°C. From the slope of the linear portion of the plots a value of 17.2 ± 0.2 kcal/mol is

obtained for the activation energy of the reaction in both cases.

The parallel lines in the Arrhenius plot suggest that the transformations of either substrate are limited at the same step in the reaction sequence. There is no indication of temperature-induced abrupt phase transitions over the whole range of temperature. It can be concluded that neither the lipid microenvironment embedding the enzyme molecule in the plasma membrane nor the phosphatidylcholine substrate undergoes a phase transition between 9°C and 44°C.

The effects of phospholipase-induced phospholipid depletion

Attempts to decrease the content of phospholipids in the plasma membranes with detergents or organic solvent extraction resulted in the irreversible loss of cholinephosphotransferase activity [5]. Phospholipases were used to decrease the amount of membrane phospholipid in order to avoid denaturation of the membrane proteins. The plasma membrane preparation was treated separately with various amounts of phospholipase A_2 from *Naja naja*, phospholipase C from *B. cereus*, phospholipase C from *C. perfringens* and phospholipase D from cabbage. A large excess of delipidated bovine serum albumin (50 mg/ml) was included in the incubation mixtures as an absorbent for any lipid material released from the membrane particles during the lipase action. After 35 min of lipolytic treatment at 22°C the lipases were removed from the membrane preparation and the cholinephosphotransferase activity as well as the changes in membrane phospholipid content were determined. Varying degrees of phospholipid loss from the membrane preparation were obtained, depending on the type and concentration of the lipase (Table II).

TABLE II

THE EFFECTS OF PHOSPHOLIPASE-INDUCED PHOSPHOLIPID DEPLETION OF PLASMA MEMBRANES ON THE CHOLINEPHOSPHOTRANSFERASE ACTIVITY

Plasma membranes from mouse Ehrlich ascites cells were incubated with the indicated amounts of the respective phospholipase at 22°C for 35 min. The membranes were washed free of the phospholipase and the residual amount of membrane phospholipid was determined. The activity of the cholinephosphotransferase in the delipidated preparations was assayed in the presence and absence of phosphatidylcholine using [^{3}H]ceramide as the labelled substrate.

Type and amount of phospholipase (units/ml incubation mixture)	Lipid P [a]	Spec. act. [a,d] cholinephosphotransferase	
		PC omitted	PC added
Phospholipase D (cabbage)			
0.15	1.00	1.00	n.d. [c]
3.00	1.00	0.52	n.d.
60.00	0.92 [b]	0.16	0.25
Phospholipase A_2 (*Naja naja*)			
0.03	1.00	0.88	n.d.
0.16	0.83	0.63	n.d.
1.00	0.73	0.52	n.d.
6.00	0.47	0.33	1.22
Phospholipase C (*B. cereus*)			
0.03	1.00	0.83	n.d.
0.16	0.77	0.62	n.d.
1.00	0.35	0.20	n.d.
6.00	0.11	0.02	0.58
Phospholipase C (*C. perfringens*)			
0.05	0.95	0.97	n.d.
0.55	0.92	0.73	n.d.
6.00	0.23	0.02	1.83

[a] Given as ratios based upon determinations in identically treated samples without phospholipases.
[b] 75% of the phospholipid fraction consisted of phosphatidic acid.
[c] n.d., not determined.
[d] The control samples had a spec. act. of 2 nmol sphingomyelin/mg protein per h.

Phospholipid D

Cabbage phospholipase D treatment removed about 8% of phospholipid material. The membrane glycerophospholipids were nearly quantitatively converted to phosphatidic acid at the highest phospholipase D concentration employed. The cholinephosphotransferase activity, as determined by the formation of radiolabelled sphingomyelin from [^{3}H]ceramide, was reduced by 80% in the sample containing 60 units of enzymes. The activity could not be restored by the presence of exogenous phosphatidylcholine in the reaction mixture (Table II).

Phospholipase A_2

Incubation with phospholipase A_2 removed about 53% of the membrane phospholipids and reduced the cholinephosphotransferase activity by

67%. In contrast to membranes treated with phospholipase D, the activity of phospholipase A_2-treated membranes could be restored with exogenous phosphatidylcholine up to 122% of the original value.

Phospholipase C

The *B. cereus* phospholipase C preparation removed 88% and the preparation from *C. perfringens* removed 77% (Table II) of the phospholipids from the membranes. There was an increase in the amount of the expected reaction products diacylglycerols and ceramides in the treated membranes. The recovery of diacylglycerol was quantitatively less than the decrease of phospholipid and absorption to bovine serum is assumed to account for this difference. In both cases, the activity of the cholinephosphotransferase was reduced to 2% of the original value; however, there were quantitative differences in the ability to restore activity with added phosphatidylcholine. The membranes that had been exposed to the *C. perfringens* phospholipase C recovered 183% activity when incubated in the presence of exogenous phosphatidylcholine in the reaction mixture. The recovery obtained for the membranes pretreated with the enzymes from *B. cereus* had an activity that was 58% of controls. This difference may be related to the almost quantitative removal of sphingomyelin from the membranes by the *C. perfringens* phospholipase C.

These results are in accord with the postulated dependence of sphingomyelin formation on the presence of phosphatidylcholine as the donor of the choline-phosphate group.

Substrate specificity

Plasma membrane particles treated with phospholipase C of *C. perfringens* were employed to test several phospholipids for their potential to act as the donor of polar head groups in the cholinephosphotransferase reaction.

Phosphatidylserine (PS), phosphatidylethanolamine (PE) and phosphatidylinositol (PI) were incubated with the treated membranes in the presence of [^{3}H]ceramide, but no reaction product, such as ceramidylphosphorylserine, could be detected. It became apparent, however, that these phospholipids were potent inhibitors of the cholinephosphotransferase reaction when exogenous phosphatidylcholine was included in the reaction mixture. PI and PE inhibited the reaction by 95% and PS decreased the reaction by 62% at 0.3 mM concentration (Fig. 3). Inhibition of the enzyme activity by PE, PI and PS was also observed in experiments with untreated plasma membranes as the source of enzyme (data not presented).

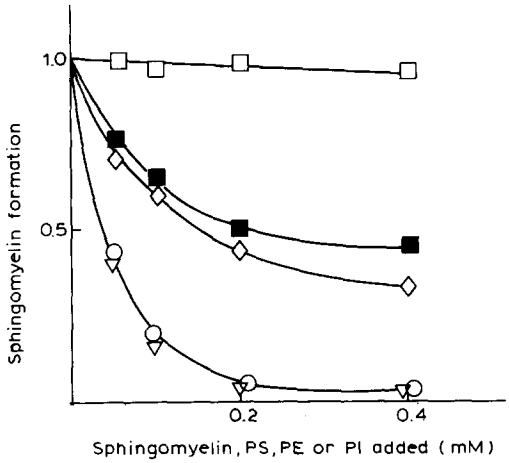

Fig. 3. Inhibition of the cholinephosphotransferase reaction of phospholipase C pretreated membranes by various phospholipids. The formation of [^{3}H]sphingomyelin was determined with fixed concentrations of phosphatidylcholine and [^{3}H]ceramide in the presence of various amounts of other phospholipids (open symbols). In the case of sphingomyelin, the formation of [^{14}C]sphingomyelin from phosphatidyl[^{14}C]-choline was also determined (closed symbols). The values are presented as ratios of control samples (spec. act. 3 nmol sphingomyelin/mg protein per h) incubated in the absence of inhibitor. □, ■, sphingomyelin; ◇, phosphatidylserine; ○, phosphatidylethanolamine; ▽, phosphatidylinositol.

Sphingomyelin was also employed except that phosphatidyl[^{14}C]choline (closed squares) utilization as substrate was also determined in addition to the utilization of [^{3}H]ceramide as the substrate (open squares). As seen in Fig. 3, [^{3}H]sphingomyelin formation from [^{3}H]ceramide is only slightly reduced by the exogenous sphingomyelin. In contrast, phosphatidyl[^{14}C]choline utilization, as determined by [^{14}C]sphingomyelin formation, is significantly reduced by the addition of sphingomyelin into the reaction mixture. This behavior can be expected on the basis of a presumptive enzyme bound phosphocholine intermediate. Both phosphati-

dylcholine and sphingomyelin can compete for the free enzyme and, therefore, act as mutual competitive inhibitors.

The existence of an enzyme bound intermediate of the transferred group is inherent to the ping-pong reaction mechanism of transfer reactions [9]. This type of reaction mechanism is favored for the cholinephosphotransferase reaction.

Specificity for the degree of fatty acid unsaturation of PC

The effect of the degree of unsaturation in the acyl chains of the phosphatidylcholine donor on the cholinephosphotransferase reaction was also tested using *C. perfringens* phospholipase-C-treated plasma membranes. Sonic dispersions of several types of synthetic phosphatidylcholine were incubated at various concentrations with a constant amount of a liposomal [^{3}H]ceramide preparation. The results presented as plots of the reciprocal of velocity against the reciprocal of phosphatidylcholine concentration indicate a preference of the enzyme for the unsaturated phosphatidylcholines (Fig. 4).

At 0.3 mM ceramide, the unsaturated phosphatidylcholines had a K_m value of 0.14 mM, which was lower than the saturated phosphatidylcholine with a range of 0.5–0.8 mM (Fig. 4). The V_{max} values are about 10-fold higher with the unsaturated species comparing the distearoyl- and dioleoylphosphatidylcholines. These could be a result of a more effective insertion of the unsaturated species in the membrane, physical state in an aqueous environment or enzyme specificity.

Determination of the K_m values for the phosphatidylcholine and the ceramide substrates

The K_m values of exogenous phosphatidylcholine for the reaction was previously determined in an assay system devoid of exogenous ceramide [6]. The reaction under those former conditions depended on the content of ceramide endogenous to the membrane particles containing the enzyme activity. A K_m of 0.025 mM was determined for phosphatidylcholine, but this value reflected a limiting supply of endogenous ceramide as subsequently became evident [8]. It was necessary, therefore, to redetermine these kinetic parameters in a system containing added ceramide in a more

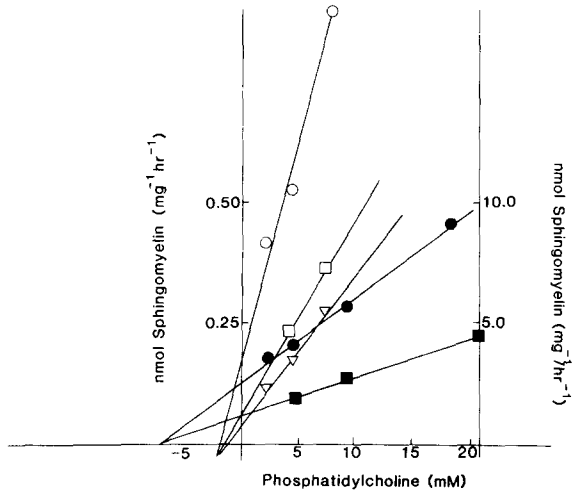

Fig. 4. Specificity of the cholinephosphotransferase for the degree of saturation in the phosphatidylcholine substrate and their kinetic constants. [^{3}H]Ceramide, 0.3 mM, was incubated with various amounts of the respective phosphatidylcholine substrate. The data for the open symbols are on the right-hand scale and the data for the closed symbols on the left-hand scale. These results were utilized to calculate the kinetic constants.

	V_{max} (nmol/mg per h)	K_m (mM)
● dioleylPC	8.1	0.14
■ dilinoleylPC	18.2	0.14
○ diarachidonoylPC	0.3	0.50
□ distearoylPC	0.8	0.60
▽ dipalmitoylPC	1.25	0.80

general context following the general techniques for the kinetic analysis of bisubstrate reactions described in ref. [9]. A set of 12 values for the initial reaction velocity of radioactive sphingomyelin formation from the combinations of four concentrations for the [^{3}H]ceramide substrate and three concentrations for the phosphatidylcholine substrate was determined. The values were corrected for the amounts of endogenous ceramide and phosphatidylcholine present in the membrane preparations.

The results are plotted as the reciprocal of the initial reaction velocity against the reciprocal of the variable substrate concentrations. The various concentrations of the respective fixed substrate provided two sets of parallel lines which are presented in Fig. 5. The intercepts with the ordinate represent the reaction velocity at infinite concentration of the variable substrate. These values

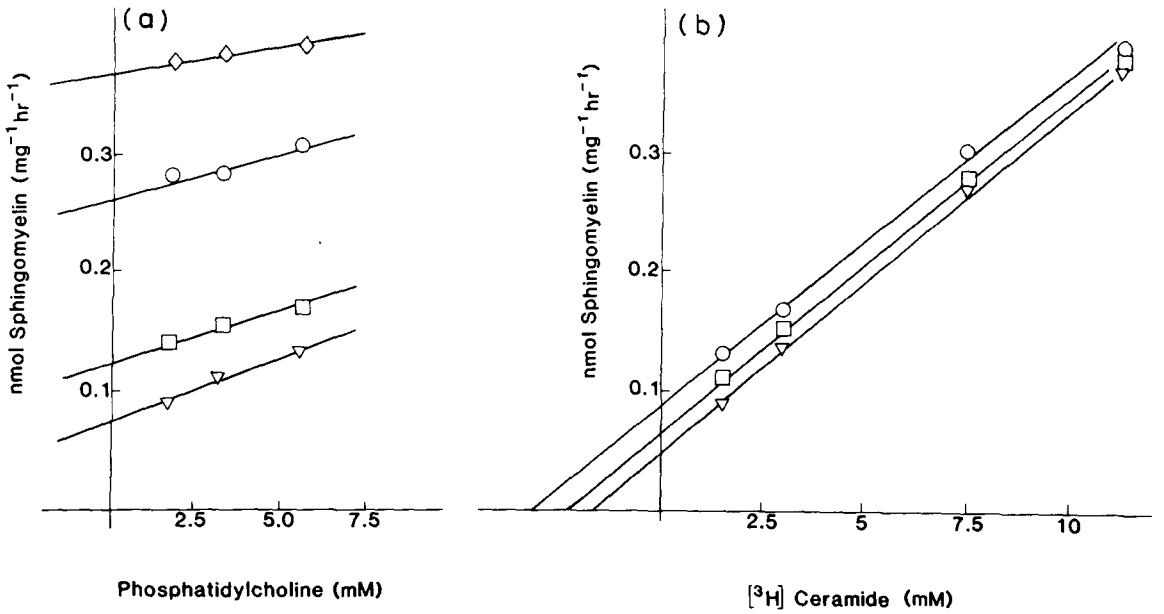

Fig. 5. (a) Double-reciprocal plot of $1/v$ versus 1/[phosphatidylcholine] at several concentrations of [^{3}H]ceramide. The values are based upon determinations of [^{3}H]sphingomyelin formation. ◇, 0.083; ○, 0.133; □, 0.333; ▽, 0.666 mM ceramide. Double-reciprocal plot of $1/v$ versus 1/[[^{3}H]ceramide] at several concentrations of phosphatidylcholine. The values are based upon determinations of [^{3}H]sphingomyelin formation. ○, 0.177; □, 0.307; ▽, 0.567 mM phosphatidylcholine.

were replotted against the reciprocal of fixed substrate concentration to give two lines with identical intercepts at $1/v$, which represents the reaction velocity at infinite concentration of both substrates (Fig. 6). The intercepts with the abscissa represent the K_m at infinite concentration of the respective fixed substrate (Fig. 6). The following values, based upon determinations with the labelled ceramide substrate, are obtained for the kinetic parameters of the cholinephosphotransferase: the K_m for ceramide is 1.0 mM and the K_m for phosphatidylcholine is 0.35 mM, and the V_{max} for the reaction is 35 nmol · mg^{-1} · h^{-1}. Because of the parallel lines seen in Fig. 5, a rate equation equivalent or identical to that of a ping-pong reaction mechanism can be postulated [9]. This reaction mechanism involves an enzyme-bound intermediate of the group being transferred and this mechanism has been postulated for several acyltransferases and of certain phosphatases [10]. The ping-pong type of reaction mechanism for the cholinephosphotransferase reaction would include two independent, consecutive steps:

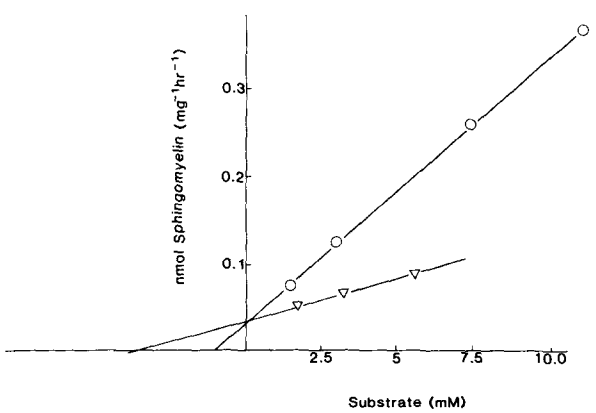

Fig. 6. Intercept replot from Fig. 5a ○ ceramide and Fig. 5b ▽ phosphatidylcholine vs. 1/[respective fixed substrate]. This is equivalent to extrapolating the kinetic parameters determined by variations of the first substrate to infinite concentration of the respective second substrate.

(1) enzyme + phosphatidylcholine → enzyme-phosphocholine + diacylglycerol

(2) Enzyme-phosphocholine + free ceramide → enzyme + sphingomyelin

It follows that this scheme, that the phosphati-

dylcholine and sphingomyelin compete for the free enzyme molecules and that ceramide and diacylglycerols compete for the enzyme-phosphocholine species. Hence, competitive inhibition between the members of the two pairs must be postulated if the reaction proceeds via a ping-pong mechanism.

Inhibition of the cholinephosphotransferase reaction by a liposomal diacylglycerol preparation

The prediction of competitivity between ceramide and diacylglycerol for the presumptive enzyme bound phosphocholine intermediate was tested measuring the reaction at various concentrations of ceramide and diacylglycerols at a constant concentration of phosphatidylcholine in the mixture. In this experiment, the formation of sphingomyelin from [^{3}H]ceramide was used for these determinations. A competitive pattern of inhibition is seen from the $1/v$ vs. $1/$[ceramide] plot (Fig. 7). The various concentrations of diacylglycerols affect only the slope, not the intercept on the ordinate, indicating that diacylglycerol inhibition does not influence the V_{max} of the reaction velocity at infinite substrate concentrations. From these data a value of 0.13 mM can be determined for the K_i of diacylglycerols. These

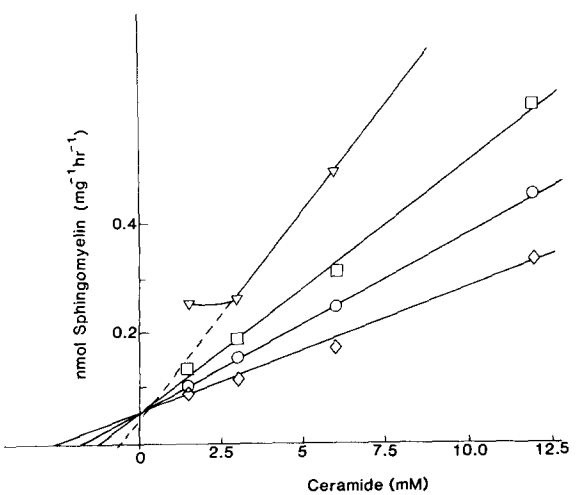

Fig. 7. Competitive inhibition of sphingomyelin formation from ceramide by diacylglycerol at a constant concentration of phosphatidylcholine (0.3 mM). The data are given as $1/v$ versus $1/$[[^{3}H]ceramide] plots at various fixed concentrations of diacylglycerols. (◇) diacylglycerols omitted; ○, 0.08; □, 0.16; ▽, 0.32 mM diacylglycerols.

results on the inhibition of the cholinephosphotransferase reaction are in accord with a presumed ping-pong mechanism. The low value of 0.13 mM for the K_i supports the well-known strong inhibitory effects of diacylglycerol on the cholinephosphotransferase reaction [3,8].

Discussion

The enzyme catalyzing the phosphatidylcholine : ceramide cholinephosphotransferase reaction is strongly bound to the plasma membrane and cannot be removed by solutions of high ionic strength or repeated washings with EDTA and it is, therefore, considered as an integral membrane protein. Association with phospholipid appears to be a prerequisite for the cholinephosphotransferase, since this is a complete loss of activity upon detergent or organic solvent treatment which cannot be restored with phosphatidylcholine replenishment [5]. This observation is in contrast with the ability to obtain complete restoration of activity with exogenous phosphatidylcholine after phospholipase-C-induced membrane phospholipid depletion. It can be assumed, that the phospholipase C treatment is a very selective attack on the membrane phospholipids with does not irreversibly modify the native conformation of the enzyme.

It follows from the intramembrane nature of the reaction that the substrates traverse this lipid phase, since the enzyme is incapable of processing substrate molecules that are not members of the membrane containing the enzyme [4,8]. The preference for the ability of phosphatidylcholines with unsaturated fatty acyl chains to restore activity observed with the phospholipid-depleted membrane preparations is consistent with a fluid state at low temperatures of the lipids surrounding the enzyme.

A ping-pong mechanism of the reaction seems appropriate for this reaction because of the bulky substrates, the restricted mobility in the ordered structure of the bilayer membrane and a reaction separated into two consecutive steps with the enzyme molecule possibly acting as the carrier of the transferred group.

Although no unusual features are apparent in Fig. 5, the interpretation is subject to some uncer-

tainty. This is due to the intramembrane mechanism of the reaction, the unknown nature of substrate movement from the liposomal preparation to the membrane particles and the movement within the plasma membrane bilayer to the enzyme.

The K_m values determined for phosphatidylcholine and ceramide may, therefore, be considered as formal analogs of their counterparts in soluble systems and to be characteristic for this type of assay system. It will be interesting to compare the values obtained with the liposomal substrates and the particulate enzyme preparation with those obtained in a fully solubilized system which may be available in the future.

The cholinephosphotransferase reaction could result in a more rigid structure in the membrane containing the enzyme due to the replacement of phosphatidylcholine molecules by sphingomyelin. The preference of the enzyme for the highly unsaturated phosphatidylcholine increases the rigidifying effect in the membranes containing a natural mixture of phosphatidylcholine.

The primary biological function of the enzyme is presumed to be the principal path for sphingomyelin biosynthesis in mammalian cells. This assumption is based upon the absence of alternative pathways for sphingomyelin [2,4] formation in most tissues and from the quantitation of the levels of cholinephosphotransferase activity in various mouse tissues. It appears that greater activity of cholinephosphotransferase activity is detected in cells having intense exchange of materials through the plasma membranes such as secretion in mammary glands and lung cells, specialized transport functions in kidney and liver cells. Plasma membrane of dividing mouse fibroblast cultures have a higher value compared to thymus or heart, but it constitutes only 27% of the value determined in liver. It can therefore be assumed that plasma membrane turnover may be an important role for the cellular sphingomyelin.

The results on the pathway of lysosomal sphingomyelin utilization in Kuppfer cells [11] gives indirect support to this hypothesis. Sphingomyelin is not released intact into the lumen of the cell but it is cleaved by a lysosomal sphingomyelinase and the choline phosphate moiety is shown to be reutilized for phospholipid synthesis. The accumulation of sphingomyelin in the cell seems to be controlled in mammalian cells. Under these conditions, plasma membrane turnover results in an increased requirement for sphingomyelin biosynthesis due to the increased loss of this lipid with the plasma membrane fraction.

The absence of activity in brain and red blood cells is surprising, as large amounts of sphingomyelin are found in these cells. The state of the red-cell membranes with its large content of sphingomyelin may constitute the result of a metabolic condition that permits the accumulation of this lipid, such as the chemical equilibrium of the reaction [7]. An alternative pathway for sphingomyelin formation has been tentatively identified in brain [5].

Nelson and Murray [12] studied the regulation of sphingomyelin content in plasma membranes of 3T3-L1 cells and found a rapid and sensitive response of the cholinephosphotransferase activity to dexamethasone for 4 h which resulted in a 50% increase of the sphingomyelin content of the plasma membranes and an 83% increase in the cholinephosphotransferase activity in a process that apparently depended on new protein synthesis. This result suggests that sphingomyelin formation in the plasma membranes of 3T3-L1 cells is kinetically controlled rather than by the chemical equilibrium of the reversible exchange reaction.

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