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In vitro synthesis and transbilayer movement of phosphatidylethanolamine molecules labelled with different fatty acids in chick brain microsomes

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The transbilayer fatty acid distribution of diacylglycerophosphoethanolamine and the translocation of newly synthesized phosphatidylethanolamine molecules labelled with different fatty acids has been investigated in chick brain microsomes using trinitrobenzensulfonic acid. The determination of the fatty acid composition of diacylglycerophosphoethanolamine in both the outer and the inner leaflet of the microsomal vesicles revealed a similar distribution indicating that both leaflets share the same molecular species. The in vitro incorporation of radioactive fatty acids (16:0, 18:1 and 20:4($n-6$)) into ethanolamine phospholipids, known to be catalyzed by the lyosphosphatidylethanolamine acyl transferase, showed that the radioactive diacylglycerophosphoethanolamine molecules appeared first in the outer leaflet and were thereafter transferred to the inner leaflet. The apparent rate of translocation of the newly synthesized ethanolamine phospholipid molecules was the highest for those labelled with 16:0 and the lowest for those labelled with 20:4($n-6$). The results indicate that the active site of the acyl-CoA:lysophosphatidylethanolamine acyltransferases is located on the outer leaflet of the microsomal vesicles and that the different newly synthesized molecular species of diacylglycerophosphoethanolamine may be translocated from the outer to the inner leaflet at different rates.

Introduction

Ethanolamine and choline phospholipids (PE and PC) account for about 70 to 80% of total brain phospholipids and, in spite of some controversial results, it becomes evident that they are distributed asymmetrically in the microsomal [1,2] and synaptosomal plasma membranes [3–5]. PE and PC constitute very heterogeneous classes of phospholipids represented by various molecular species which differ in the structure and composition of their fatty acids. Recent investigations have shown that diacyl-GPE and diacyl-GPC are constituted of about 15–18 major molecular species each [6,7]. The analysis of the fatty acid composition of synaptosomal plasma membrane PE revealed that the inner leaflet contains more unsaturated fatty acids than the external leaflet hence suggesting also an asymmetric distribution of PE molecular species in the membrane [5,8]. These observations raised the question on the nature of the mechanisms involved in the asymmetric assembly of membrane phospholipids.

The synthesis of phospholipids in the nervous system occurs mainly in the endoplasmic reticulum [9–12]. In this membrane structure PE is mostly situated in the luminal leaflet whereas PC is mainly located in the cytoplasmic leaflet [1,2]. The synthesis de novo of both phospholipids takes place on the cytoplasmic leaflet [13–15] and a translocation to the luminal surface is then required for the establishment of the bilayer asymmetry. Studies on the transbilayer movement of phospholipids in microsomal vesicles have shown that the rate of translocation from the cytoplasmic to the luminal leaflet, is much higher for PE molecules than for PC molecules in agreement with their asymmetric distribution [15]. However, nothing is known on the mecha-

Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; diacyl-GPE, diacylglycerophosphoethanolamine; diacyl-GPC, diacylglycerophosphocholine; TNBS, 2,4,6-trinitrobenzenesulfonic acid; TNP-diacyl-GPE, *N*-2,4,6-trinitrophenyl-diacylglycerophosphoethanolamine.

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nisms controlling the transbilayer movements of molecular species of phospholipids with respect to their fatty acid composition in neuronal membranes. The present report concerns the investigation of the fatty acid distribution in the diacylglycerophosphoethanolamine of both leaflets in chick brain microsomal vesicles and the transbilayer movement of newly synthesized molecules labelled with different fatty acids.

Materials and Methods

Materials

(1-^{14}C)-labelled fatty acids (16:0 (spec. act., 56 mCi/mmol), 18:1 (spec. act., 57.4 mCi/mmol), 20:4(n − 6) (spec. act., 53.9 mCi/mmol)) were purchased from Amersham International U.K. ATP, CoASH, Phospholipase A_2 from *Vipera russelli* and bovine serum albumin were obtained from Sigma Chemical (St Louis, MO, USA) and TNBS from Polysciences (Northhampton, U.K.). All other reagents were of the highest purity grade.

Preparation of microsomes

Microsomes from adult chicken brain were prepared and their purity assessed as described by Porcellati et al. [10] with slight modifications as reported by Dominski et al. [1]. The microsomes were homogenized in appropriate buffer and used without storage.

Labelling of microsomal phospholipids

Chick brain microsomes were labelled with different fatty acid through the lysophospholipid acyltransferase pathway. Microsomes (1.8 mg protein) were incubated at 37 °C in 40 mM Hepes buffer (pH 7.4) containing 150 mM NaCl, 10 mM $MgCl_2$, 10 mM ATP, 0.2 mM CoASH and 0.05 mM ^{14}C-labelled fatty acid (spec. act., 53.9 mCi/mmol) in a final volume of 0.35 ml. The reaction was stopped at various time intervals by cooling the samples to 0 °C.

Reaction of ethanolamine phospholipids with TNBS

At the end of the incubation with radioactive fatty acids, microsomes were rapidly reacted with TNBS at 0 °C which labels the PE and PS molecules of the outer leaflet as reported by Dominski et al. [1]. Briefly 1.65 ml of 170 mM $NaHCO_3$ (pH 8.0) were added to each sample followed by 0.25 ml of 15 mM TNBS in 5% $NaHCO_3$ adjusted to pH 9.00. The reaction lasted for 20 min at 0 °C and was stopped by the addition of 1 ml of ice-cold 2.5 M perchloric acid. The suspension was centrifuged at 2500 × g for 30 min and the supernatant was discarded. The pellet was washed once with 1 ml of 0.6 M perchloric acid. The treatment of microsomes with perchloric acid produced the hydrolysis of plasmalogens into lysophospholipids [13], therefore both the unreacted ethanolamine phospholipids and those reacted with TNBS corresponded to diacylglycerophosphoethanolamine.

Lipids were extracted from the microsomal pellet with 2 ml of a mixture of chloroform/methanol (2 : 1, by vol.) as described by Folch et al. [16]. The lipid extract was washed with 0.4 ml 125 mM NaCl and 0.4 ml of the theoretical upper phase successively. The organic phase was evaporated under a nitrogen stream.

Phospholipids were separated by two-dimensional thin-layer chromatography using Silica gel G-60 plates (Merck) and developed with chloroform/methanol/27% ammonia (65 : 35 : 5, by vol.) in the first dimension and chloroform/methanol/acetone/acetic acid/water (75 : 15 : 30 : 15 : 7.5, by vol.) in the second. Phospholipids were visualized with iodine vapours and the spots corresponding to diacyl-GPE and TNP-diacyl-GPE scraped and analyzed for phosphate content and radioactivity. For the determination of the diacyl-GPE fatty acid composition the plates were stained with primuline [17].

Analysis of the fatty acid composition of ethanolamine phospholipids

The fatty acid distribution of diacyl-GPE and TNP-diacyl-GPE was performed by GLC as reported by Pelletier et al. [17]. To estimate the position of the fatty acid on the glycerol moiety (C1 or C2), the diacyl-GPE and TNP-diacyl-GPE were eluted from the silica gel with 5 ml of chloroform/methanol (2 : 1, by vol.) and 5 ml of methanol. After evaporation of the solvent the samples were treated with phospholipase A_2 from *Vipera russelli* [18]. The diacyl-GPE and TNP-diacyl-GPE were emulsified in 1 ml of 20 mM Tris-HCl (pH 7.4), 5 mM $CaCl_2$, 0.1% bovine serum albumine. 50 mU of phospholipase A_2 were added and the samples incubated at 37 °C for 30 or 60 min. The reaction was terminated by adding 0.2 ml of 1 M HCl and the lipids extracted with 6 ml of chloroform/methanol (2 : 1, by vol.). After evaporation of the organic phase the fatty acids and the lysophosphatidylethanolamine compounds were separated by thin-layer chromatography on Silica gel G60 plates with chloroform/methanol/acetone/acetic acid/water (75 : 15 : 30 : 15 : 7.5, by vol.). After visualization with iodine vapours the spots corresponding to the fatty acid, lysophosphatidylethanolamine and TNP-lysophosphatidylethanolamine were scraped into counting vials for determination of the radioactivity.

Proteins were determined according to Lowry et al. [19] and the lipid phosphorus by the method of Rouser et al. [20]. The radioactivity was measured with 10 ml Biofluor (Du Pont de Nemours) and 0.5 ml water in a Packard Tri Carb 4000 scintillation spectrometer. The amount of fatty acids incorporated into diacyl-GPE was calculated from the specific activity of the precursors measured under the same conditions.

Results

Fatty acid composition of diacyl-GPE in the outer and inner leaflet of microsomal vesicles

The distribution of fatty acids in diacyl-GPE of both microsomal leaflets has been determined using TNBS in non penetrating conditions. As reported elsewhere the pool of diacyl-GPE available to TNBS and that unavailable to the probe represented, respectively, the molecules located in the outer and inner leaflets of the microsomal vesicles [1]. No significant differences were observed in the fatty acid composition of diacyl-GPE present in both the outer and inner leaflets (Table I). In both leaflets diacyl-GPE contained about 50% of saturated (mainly 16:0 and 18:0) about 17% of monounsaturated (mainly 18:1) and about 33% of polyunsaturated (mainly 20:4($n-6$) and 22:6($n-3$)) fatty acids. This distribution was similar to that obtained for whole microsomal diacyl-GPE and are in agreement with the fatty acid distribution reported for whole chick brain PE [21].

In vitro incorporation of fatty acids into diacyl-GPE of microsomal vesicles

The incubation of microsomes with radioactive fatty acids showed that 20:4($n-6$) was incorporated at a higher rate into diacyl-GPE than 18:1 or 16:0 (Fig. 1). 20:4($n-6$) was incorporated linearly for about 20 min and at a slower rate thereafter. Similar results were obtained for 18:1($n-9$) but the incorporation after 60 min of incubation was lower than that of 20:4($n-6$) and corresponded to 65% of it. 16:0 was much less incorporated into diacyl-GPE than the two unsaturated fatty acids. Its incorporation was linear for about 10 min and reached a plateau after 15 min. The amount incorporated after 60 min accounted for about only 16% of that of 20:4($n-6$).

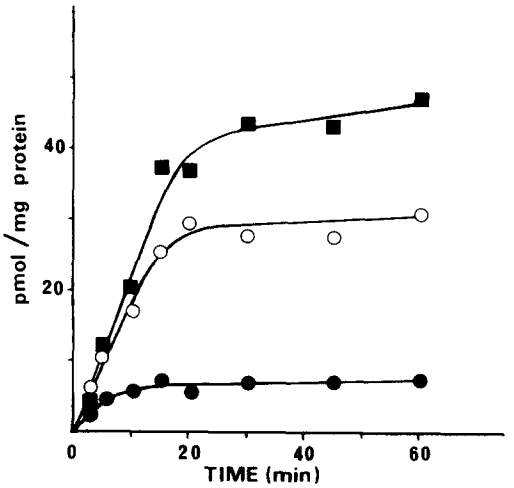

Fig. 1. Incorporation of fatty acids into diacylglycerophosphoethanolamine of chick brain microsomes. Microsomes were incubated with radioactive 16:0 (●——●), 18:1 (○——○) or 20:4($n-6$) (■——■) as described in Materials and Methods. Each point represents the average of duplicate determinations of two different experiments. The standard deviation was less than 10%.

The determination of the position esterified by the various fatty acids in the glycerol moiety of the diacyl-GPE molecules during the incubation revealed that 16:0 was preferentially incorporated into the C1 position and 20:4($n-6$) preferentially into the C2 position whereas 18:1 shared equally positions C1 and C2 (Table II). Similar results were obtained when microsomes were incubated for 30 or 60 min with the radioactive fatty acids.

Side of fatty acids incorporation into microsomal diacyl-GPE and translocation of the newly synthesized molecules

In order to obtain information on the localization of acyl-CoA:lysophosphatidylethanolamine acyltransfer-

TABLE I

Fatty acid distribution in diacylglycerophosphoethanolamine of the outer and inner leaflet of chick brain microsomes

Microsomes were treated with TNBS for 20 min at 0 °C. The TNP-diacyl-GPE and diacyl-GPE were separated by thin-layer chromatography and the fatty acid composition analysed by gas-liquid chromatography as described in Materials and Methods. Values ± S.E. represent the average of three different preparations.

Fatty acids	Outer leaflet	Inner leaflet
14:0	2.00 ± 0.32	1.22 ± 0.42
16:0	16.20 ± 1.54	15.39 ± 3.81
17:0	1.58 ± 0.38	1.54 ± 0.47
18:0	31.45 ± 2.59	26.83 ± 2.02
16:1($n-9$)	2.22 ± 0.43	1.30 ± 0.30
18:1($n-9$)	11.75 ± 1.81	15.74 ± 0.78
20:3($n-9$)	1.70 ± 0.34	1.92 ± 0.11
18:1($n-7$)	1.70 ± 0.15	1.04 ± 0.10
18:2($n-6$)	0.96 ± 0.12	0.67 ± 0.06
20:4($n-6$)	10.22 ± 3.29	9.91 ± 0.42
22:4($n-6$)	4.20 ± 1.34	5.00 ± 0.36
22:5($n-6$)	5.22 ± 1.55	5.99 ± 0.43
22:6($n-3$)	9.94 ± 2.21	10.81 ± 0.66

TABLE II

Incorporation of radioactive fatty acids into diacylglycerophosphoethanolamine of the outer and inner leaflet of chick brain microsomal vesicles. Distribution of the radioactivity in position C1 and C2 of the glycerol

Microsomes were incubated with radioactive fatty acids for 30 min and treated as described in Materials and Methods. The results are expressed in % of fatty acid incorporated in position 1 and 2 of the glycerol, and represent the average of two experiments.

Fatty acid incorporated	TNP-diacyl-GPE		diacyl-GPE	
	C1	C2	C1	C2
16:0	75.0	25.0	71.0	29.0
18:1($n-9$)	51.0	49.0	52.0	48.0
20:4($n-6$)	17.0	83.0	19.0	81.0

ases and the translocation of the newly synthesized diacyl-GPE molecules, microsomes were incubated with radioactive fatty acids for various time periods, and thereafter reacted with ice cold TNBS.

After 30 and 60 min of incubation the distribution of the radioactive fatty acids in the position C1 and C2 of the glycerol moiety was similar in the diacyl-GPE reacting or not with TNBS (Table II). Under the present experimental conditions, the results do not indicate any rearrangement of the fatty acids in diacyl-GPE molecules during their translocation.

In microsomes incubated with radioactive 16:0 for 3 min the amount of the fatty acid incorporated into the diacyl-GPE of the outer leaflet was about 2.5-times higher than that of the inner leaflet (Fig. 2A). The incorporation of 16:0 into this phospholipid in both leaflets raised rapidly during the first 10 min and reached thereafter a plateau in the outer leaflet while it continued to increase very slightly in the inner one.

The ratio (16:0 diacyl-GPE external)/(16:0 diacyl-GPE internal) decreased rapidly from 2.5 to about 1.0 between 3 and 15 min and at a slower rate thereafter up to 60 min.

The incubation of microsomes with radioactive 18:1 led also to the synthesis of radioactive diacyl-GPE. As for 16:0 much more radioactive diacyl-GPE was found in the outer than in the inner leaflet after 3 min of incubation (Fig. 2B). Between 3 and 60 min the incorporation of 18:1 into the external diacyl-GPE increased linearly for about 20 min and reached a plateau thereafter. During the same period of incubation the incorporation of 18:1 into the diacyl-GPE of the inner leaflet

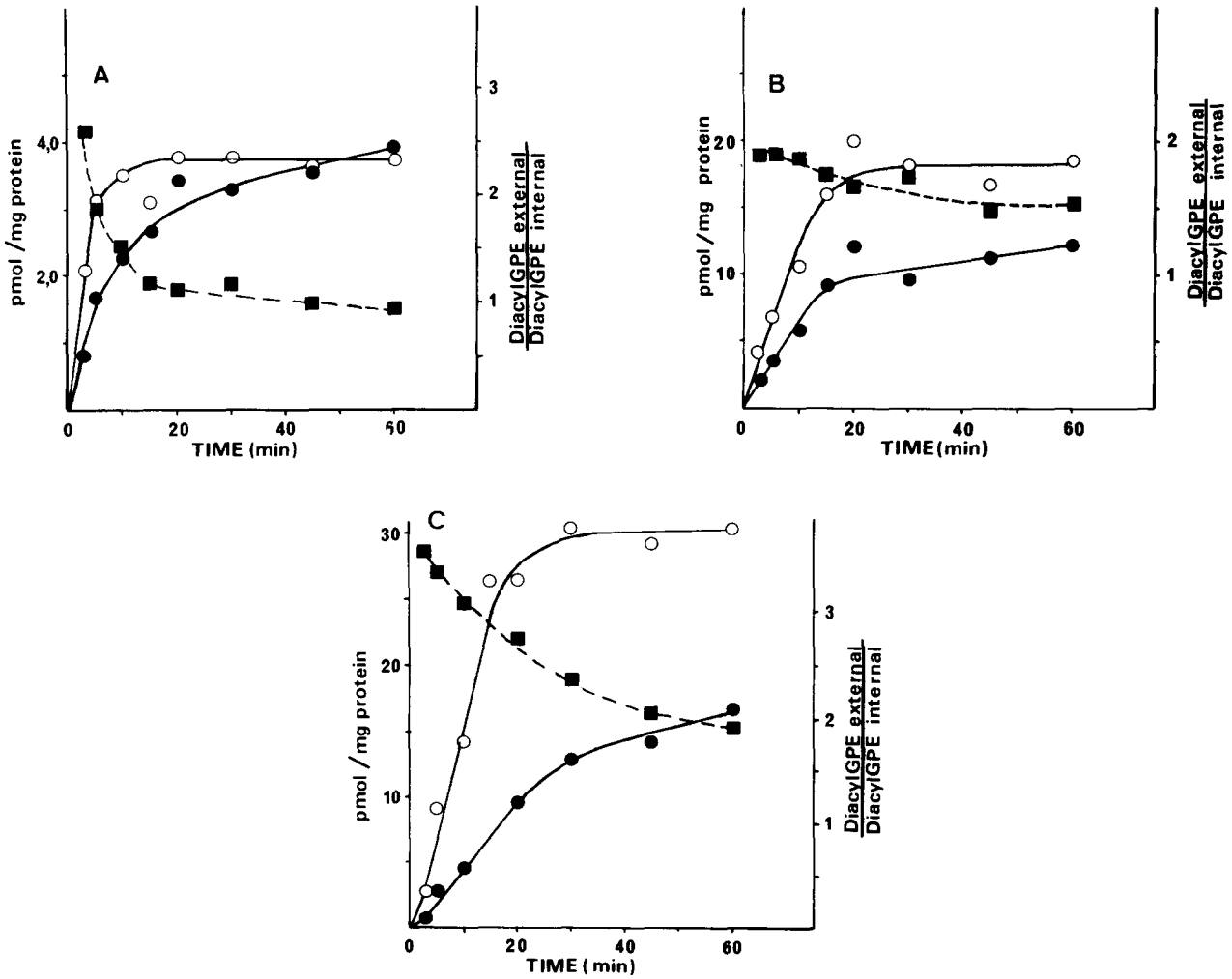

Fig. 2. Incorporation of (A) 16:0, (B) 18:1 and (C) 20:4($n-6$) into the diacylglycerophosphoethanolamine of the outer (○———○) and inner (●———●) leaflets of chick brain microsomes. Microsomes were incubated with ^{14}C-labelled fatty acids for various time periods, and then treated with TNBS as described in Materials and Methods. ■- - - - -■, ratios of the amount of the fatty acids incorporated into diacylGPE in the outer and inner leaflets. Each point represents duplicate determinations of two different experiments. Standard deviation was less than 10%.

also increased linearly during the first 20 min and continued to increase at a slower rate up to 60 min. The ratio (18:1 diacyl-GPE external)/(18:1 diacyl-GPE internal) decreased slowly from 1.90 to 1.50 between 3 and 60 min.

The incorporation of 20:4($n-6$) into the diacyl-GPE of both microsomal leaflets is reported in fig. 2C. After a short time of incubation (3 min), the incorporation of this fatty acid into the outer leaflet diacyl-GPE was about 3.5-times higher than that observed in the inner one. With time the amount of 20:4($n-6$) incorporated into this phospholipid in the outer leaflet increased linearly for about 20 min and reached a plateau after 30 min. During the period of incubation the incorporation of 20:4($n-6$) into the diacyl-GPE of the inner leaflet also increased. However the rate of increase in this leaflet was much higher than that in the outer leaflet as can be inferred from the ratio (20:4 diacyl-GPE external)/(20:4 diacyl-GPE internal) which decreased from about 3.5 to 1.8 between 3 and 60 min.

Discussion

Recent studies on chick brain microsomal membranes have indicated an asymmetric distribution of the phospholipids [1]. In these vesicles about 70% of the ethanolamine phospholipids are located in the inner leaflet. The present results showed no significant differences in the fatty acid composition of the diacyl-GPE in the inner and outer leaflets. A similar distribution of fatty acids in the PC molecules of the both leaflets has also been reported [22]. These results suggest that in chick brain microsomes both leaflets share the same molecular species of PE and PC and that the phospholipid asymmetry in these membranes may be restricted to the polar headgroup.

The synthesis of ethanolamine phospholipids through the Kennedy or the base exchange pathways takes place on the outer leaflet of the microsomal vesicles [15,23–25] and the newly synthesized molecules are translocated to the inner leaflet. Their translocation is energy dependent (unpublished results) and may be regulated by divalent cations [26]. In order to obtain an insight on the translocation of the molecular species of PE in brain microsomal membranes, the distribution of the newly synthesized diacyl-GPE molecules, labelled in vitro with various radioactive fatty acids through the lysophospholipid acyl transferase pathway, was determined in both leaflets.

The incubation of microsomes with radioactive fatty acids showed that 16:0 was preferentially incorporated into position C1 of the glycerol and 20:4($n-6$) essentially into position C2 whereas 18:1 was equally distributed between carbon C1 and C2 (Table II). Such specificity of the esterification of lysophospholipids by various fatty acid has also been reported for liver microsomes [27] and suggests that chick brain microsomes contain at least two different acyl-CoA: lysophosphatidylethanolamine acyltransferases: one esterifying the 2-acyl-GPE specific for 16:0 and 18:1 and the other esterifying the 1-acyl-GPE specific for 20:4 and 18:1.

In vitro incorporation of 16:0, 18:1 and 20:4($n-6$) into microsomal diacyl-GPE led to the synthesis of various molecular species. Recent studies revealed that 16:0 is mainly incorporated into 16:0/22:6, 18:1 into 18:1/18:1 and 18:0/18:1 and 20:4 into 18:0/20:4 diacyl-GPE (data not shown). These results are consistent with the observations that in diacyl-GPE from brain homogenates and microsomes 16:0 is essentially coupled with 22:6, 18:1 with 18:1 and 18:0 and that 20:4 is paired mainly with 18:0 [6,7]. It can thus be assumed in a first approximation that the diacyl-GPE labelled with 16:0 or 20:4 allows to follow the translocation of 16:0/22:6 and 18:0/20:4, respectively. For the molecules labelled with 18:1 the translocation will refer essentially to two molecular species mainly 18:1/18:1 and 18:0/18:1 diacyl-GPE which may behave differently and the apparent rate of transfer observed may be an average of the rates of the two molecular species. Regardless the structure of the molecular species of diacyl-GPE synthesized in the present experiments their transbilayer movement occurred without any rearrangement of the fatty acids, because the distribution of the radioactive fatty acids in both C1 and C2 positions of the glycerol was similar in the diacyl-GPE molecules of both the inner and outer leaflet.

When microsomes were shortly incubated with different radioactive fatty acids (3 min) the amount of radioactive diacyl-GPE in the outer leaflet was about 2–3.5-times higher than that in the inner leaflet. These results suggest that the catalytic site of the acyl-CoA:lysophosphatidylethanolamine acyltransferases may be located in the outer leaflet of the microsomal vesicles which corresponds to the cytoplasmic side in situ [1]. This hypothesis is in agreement with the observations reported for liver microsomes indicating that the synthesis of phospholipids takes place on the cytoplasmic leaflet [23–25].

Once synthesized in the outer leaflet, the different diacyl-GPE molecules are translocated to the inner leaflet but the apparent rate of translocation depends on the fatty acid incorporated as it can be inferred from the ratios (diacyl-GPE external)/(diacyl-GPE internal) which gives an indication on the apparent rate of transfer of the molecules from the outer to the inner leaflet. These ratios decreased differently with time for the molecules labelled with different fatty acids. These different rates of decrease for the newly synthesized molecule of diacyl-GPE may reflect either: (a) a higher rate of translocation of the molecules labelled with 16:0 than those labelled with 18:1 and 20:4($n-6$) or (b) a lower rate of synthesis of the former molecules. However

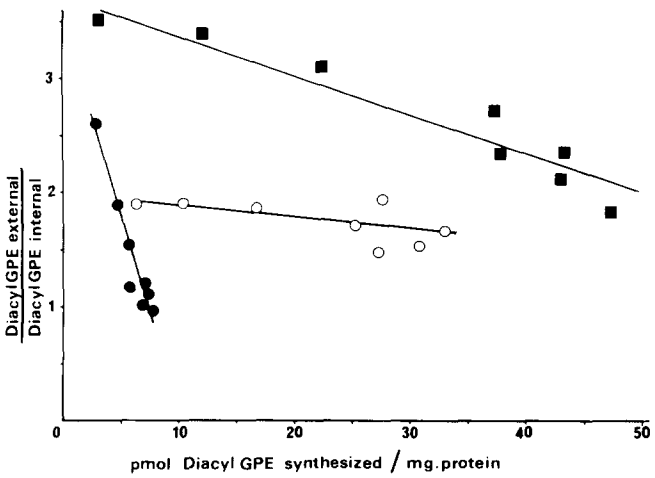

Fig. 3. Ratios of the amount of fatty acid incorporated into diacylglycerophosphoethanolamine in the outer and inner leaflets of chick brain microsomes as a function of the amount of newly synthesized diacylglycerophosphoethanolamine. 16:0 (●——●), 18:1 (○——○) and 20:4($n-6$) (■——■).

when the ratio (diacyl-GPE external)/(diacyl-GPE internal) is expressed as a function of the amount of newly synthesized molecules (Fig. 3) it may be observed that the PE molecules labelled with 16:0 are translocated at a much higher apparent rate from the outer to the inner leaflet than those labelled with 20:4($n-6$). The molecules labelled with 18:1 being translocated at an intermediate rate may be the resultant of fast and slow transferred molecular species, because this fatty acid was equally incorporated into position 1 and 2 of the glycerol moiety and mainly labelled two molecular species. The data indicate that the apparent rate of transfert of PE molecules synthesized through the lysophosphatidylethanolamine acyltransferase pathway depends on their fatty acid composition and suggest that the different newly synthesized molecular species of diacyl-GPE may be translocated at different rates. Different rates of translocation for various molecular species of phosphatidylcholine have also been reported in erythrocyte plasma membranes by Van Meer and Op den Kamp [28] and Middelkoop et al. [29].

The mechanisms which control the translocation of different molecular species of PE in biological membranes are still unknown. Recent studies showed that in plasma membranes the transbilayer movement of PE molecules is energy dependent [30,31] and suggest that it may be catalyzed by an aminophospholipid translocase or flippase [31,32]. At present it is only possible to speculate on the mechanisms involved in the control of the translocation of the various molecular species of PE in chick brain microsomes. The different rates of translocation observed may be a consequence of the molecular geometry of the various PE molecules, which may influence their interaction with the flippases. Alternatively they may reflect the heterogeneity of different domains in the lateral plane of the membrane. In this respect it may be possible that the site of synthesis of the various molecular species of PE is not located in the same area than the proteins catalysing the transbilayer movements. This hypothesis is consistent with the assumption that the active site of the acyl-CoA:1-acyl-GPE acyltransferase and the acyl-CoA:2-acyl-GPE acyltransferase reside on different proteins which may be located at different distances from the site of translocation. This hypothesis suggests that the site of translocation of the PE molecules belongs to specific domains in the lateral plane distinct from those involved in the synthesis of these molecules.

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