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INCORPORATION OF *CIS*-PARINARIC ACID, A FLUORESCENT FATTY ACID, INTO SYNAPTOSOMAL PHOSPHOLIPIDS BY AN ACYL-CoA ACYLTRANSFERASE

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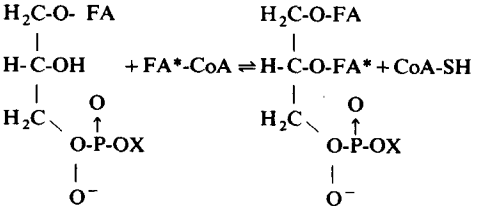
The *cis*-isomer of parinaric acid, a naturally occurring C-18 polyene fatty acid, was incubated with brain subcellular fractions and the polarization of fluorescence increased in a time dependent manner. Greatest increases occurred in synaptosomal and microsomal membranes. This increase in polarization of fluorescence was found with the *cis*, but not the *trans*, isomer of parinaric acid and required Mg^{2+} or Ca^{2+} and was stimulated by coenzyme A and ATP. Synaptosomes were incubated with *cis*-parinaric acid and lipids were extracted and examined by high performance liquid chromatography. The highest incorporations of *cis*-parinaric acid were found in phosphatidylcholine (71%) and phosphatidylethanolamine (20%) while only traces were found in phosphatidylserine and phosphatidylinositol. [^{3}H]Oleic acid was also incorporated into membrane phospholipids and unlabeled oleic acid blocked incorporation of *cis*-parinaric acid. It is proposed that *cis*-parinaric acid, like fatty acids normally found in brain, is incorporated into membrane phospholipids by an acyl-CoA acyltransferase. The presence of this enzyme in nervous tissue may make it possible to easily introduce fluorescent fatty acid probes into membrane phospholipids and to thereby facilitate study of membrane-mediated processes.

Introduction

In the course of examining the interactions of fluorescent fatty acids with synaptosomal membranes it was observed that a steady-state polarization of fluorescence was not achieved. We suspected that incorporation of fluorescent fatty acid into phospholipids via an acyltransferase might be involved. This enzyme was previously found in rat liver by Lands [1] and has subsequently been found in brain by several investigators [2,3]. The reaction involves transfer of a fatty acid (FA) into the C-1 or C-2 positions of a lysophospholipid after initial activation of the free fatty acid (FFA) via reaction (1).

$$\text{FFA} + \text{MgATP} + \text{CoA-SH} \rightleftharpoons \text{FA-CoA} + \text{PP}_i + \text{AMP} \qquad (1)$$

followed by the transferase reaction (2), where X represents different phospholipid bases:



(2)

Abbreviations used: FA, fatty acid; FFA, free fatty acid; HPLC, high performance liquid chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; TLC, thin-layer chromatography.

The technical difficulties of working with a membrane associated enzyme system and usually with a water-insoluble substrate and products are obvious. The usual procedures that have been used to study the enzyme have involved incubation of isolated membranes containing the associated enzyme with a radioactive fatty acid or fatty acyl-CoA derivative in the presence of a specific lysophospholipid. This is followed by total lipid extraction, identification and quantitation of products by thin-layer chromatography. The present study is an attempt to characterize the process responsible for the changes of polarization of fluorescence seen when synaptosomes are incubated in the presence of the naturally fluorescent fatty acid, parinaric acid, and is directed toward development of an alternative rapid assay for fatty acyl-CoA acyltransferase activity.

The polarization of fluorescence is a physical measure of the freedom of rotation the fluorophore possesses within its environment. For example, if the fluorescent compound were dissolved in an isotropic solvent and free to rotate about all axes, the polarization of fluorescence would approach zero. If the same compound were held rigidly in the plane of the exciting light, the polarization value would approach 0.5, the theoretical maximum. This fluorescent parameter is independent of the fluorophore's concentration, assuming that there is no interaction between fluorescent molecules, and is independent of fluorescent intensity. In the case of the long linear fluorescent molecule parinaric acid (Fig. 1) in a biological membrane, the polarization of fluorescence is a measure of the fatty acid's rotation within the plane of the membrane. Increasing polarization of fluorescence indicates restricted rotation of the molecule. The two isomers of parinaric acid are thought to partition into different regions of the lipid bilayer [4], the *cis*-isomer going into the more fluid areas while the *trans*-isomer of parinaric acid probes the less fluid or organized regions.

Preliminary work had been done in our laboratory using both isomers of parinaric acid on red blood cell ghosts, intact human fibroblasts, plasma membranes of those fibroblasts, and intact lymphocytes. In these membrane systems, a steady polarization of fluorescence of either isomer of the fatty acid was observed 90 s after addition of the probe when the experiment was run at ambient or higher temperatures. On the other hand when *cis*-parinaric acid was incubated with rat brain synaptosomes a time-dependent increase in polarization of fluorescence was found. Evidence is presented that an acyltransferase is present in synaptosomes which incorporates the fluorescent fatty acid into membrane phospholipids.

Materials and Methods

Synaptosomes were prepared by the method of Cotman et al. [5] using a 10% homogenate of rat brain in 0.32 M sucrose. Synaptosomes were collected as the material banding in a discontinuous sucrose gradient between the 0.8 and 1.2 M sucrose layers. Myelin banded between the 0.32 and 0.8 M sucrose layers while mitochondria pelleted in 1.2 M sucrose. The nuclear fraction was pelleted at $600 \times g$ for 10 min and microsomes were pelleted at $36\,000 \times g$ for 1 h. Protein content was measured by the method of Lowry et al. [6].

Human red blood cell ghosts were prepared by method of Dodge et al. [7].

Phosphate-buffered saline (comprised 0.9% NaCl and 0.10 M sodium phosphate, pH 7.4.

Cis- and *trans*-parinaric acids were obtained from Molecular Probes, Plano, TX. The concentrations of these fluorescent fatty acids were determined using the molar extinction coefficient in ethanol of 78 000 $M^{-1} \cdot cm^{-1}$ for *cis*- and 89 000 $M^{-1} \cdot cm^{-1}$ for *trans*-parinaric acid [4]. Stock solutions of both isomers were prepared in ethanol with butylated hydroxytoluene as an anti-oxidant at a molar ratio of 0.5 M butylated hydroxytoluene: 1 M parinaric acid. The parinaric acid was examined for oxidation using Merck silica gel thin-layer chromatography (TLC) plates containing fluorescent indicator and developed with pentane/diethyl ether/acetic acid (80 : 20 : 1, v/v). Parinaric acid appeared as a dark spot viewed with short wavelength ultraviolet light with an R_F of 0.42 while the oxidized material remained at the origin.

Polarization of fluorescence was measured in a SLM 4800 subnanosecond Fluorometer equipped with Glan-Thompson Calcite prism polarizers, and a thermostatted cell compartment that was maintained in a nitrogen atmosphere. The sample was

continuously stirred during analysis. The ratio of the fluorescence intensity of perpendicular and parallel polarizers was sampled a minimum of 10 times at 0.25 s per sample for each reading.

The compositions of the incubation media are indicated in the legends to the figures or tables. Typically synaptosomes in 2 ml of buffer, given in the legend to each table or figure, were thermally equilibrated in the fluorometer at 37°C (monitored by a thermistor probe, Texas Instruments, within the suspension). After 5 min at 37°C, 5 μl of *cis*-parinaric acid in ethanol was added and mixed. Polarization and fluorescent intensity were measured at the stated times. Light scattering by the synaptosomal preparation at the concentrations used contributed between 5 and 8% of the total signal. This scattered signal was constant at the times the data was collected. Because changes in fluorescent polarization were being compared, a correction for light scattering was not made.

For high performance liquid chromatography (HPLC) experiments, large scale preparations consisting of 1–2 mg of synaptosomes in 250 mM Tris-HCl buffer, pH 7.4, 3 mM ATP, 5 mM $MgCl_2$ and 0.1 mM coenzyme A were incubated at 37°C under nitrogen for 90 to 120 min. Lipid extraction was performed either by the procedure of Folch et al. [8] or by the method of Conner et al. [9] as indicated. For the removal of non-acylated parinaric acid by use of bovine serum albumin the following procedure was followed. After the incubation at 37°C (described above) an aliquot containing synaptosomes and *cis*-parinaric acid was mixed with an equal volume of bovine serum albumin (10 mg/ml) in the identical buffer. As a control, a second synaptosomal sample was added to buffer. Both were incubated 30 min at 37°C, then centrifuged at 20000 rpm in a Spinco 40 rotor and washed two times to remove bovine serum albumin.

HPLC was carried out by the method of Chen and Kou [10] using a Waters (Milford, MA) liquid chromatograph. A Waters μ-Porasil (silica) column 3.9 × 30 cm was used with a solvent system of acetonitrile/methanol/85% phosphoric acid (130 : 5 : 1.5, v/v), with a flow rate of 1 ml/min. Detection was by absorbance measurement at either 203 nm or 319 nm or fractions were collected and radioactivity measured in a Packard Tri-Carb 460C Scintillation Counter. The column was equilibrated with a solvent for a minimum of 120 min prior to the first analysis.

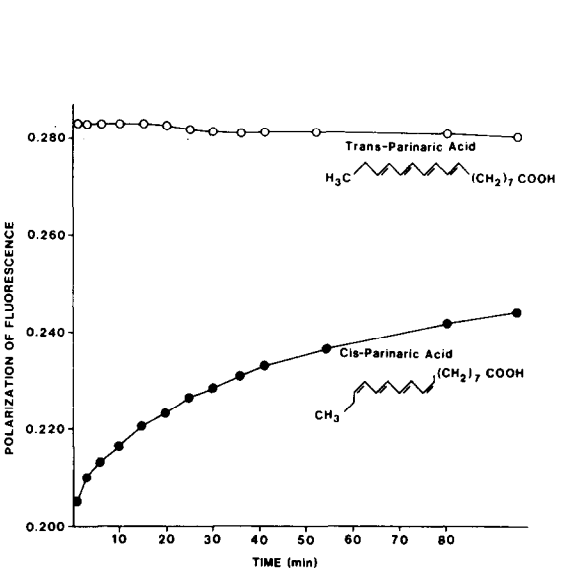
To determine the identity of each HPLC peak, phospholipid standards (Supelco Co, Bellefonte, PA) were injected and the retention times measured. A disadvantage of this solvent system is that the aqueous phosphoric acid causes progressive deactivation of the silica column. Retention times for the individual phospholipids species became longer as the sequential analysis progressed, but the relative position of each phospholipid peak was constant.

To confirm the identity of the parinoyl-phosphatidylcholine peak on HPLC, samples from that peak were collected. The phosphoric acid was neutralized with NaOH and the sample volume reduced under N_2. The phospholipid was extracted into the organic phase of a chloroform/water system. The phospholipids were chromatographed on Merck Silica Gel G plates in a solvent system chloroform/methanol/acetic acid/water (65 : 25 : 4 : 8, v/v). Phosphatidylcholine was identified by use of Dragendorf spray for choline.

Results

Factors affecting polarization of fluorescence

A steady rise in the polarization of fluorescence was observed when *cis*-parinaric acid was added to a suspension of rat brain synaptosomes at 37°C (Figs. 1 and 2). Within 90 min the polarization of fluorescence of *cis*-parinaric acid had increased by 20%. By contrast, *trans*-parinaric acid had a higher initial polarization value which decreased by 2% after 90 min. This difference in polarization between *cis*- and *trans*-parinaric acid is thought to reflect the difference in location of each isomer within biological membranes [4]. As shown in Tables I and II, all other brain subcellular fractions examined also mediate an increase in polarization of *cis*-parinaric acid, but not as great a change was observed as in synaptosomes. Red blood cell ghosts incubated with *cis*-parinaric acid show no change in polarization with time and the polarization of the *trans*-isomer of parinaric acid maintained a constant value in each of the membranes examined (Table I). In other experiments, phospholipids were first extracted from membranes



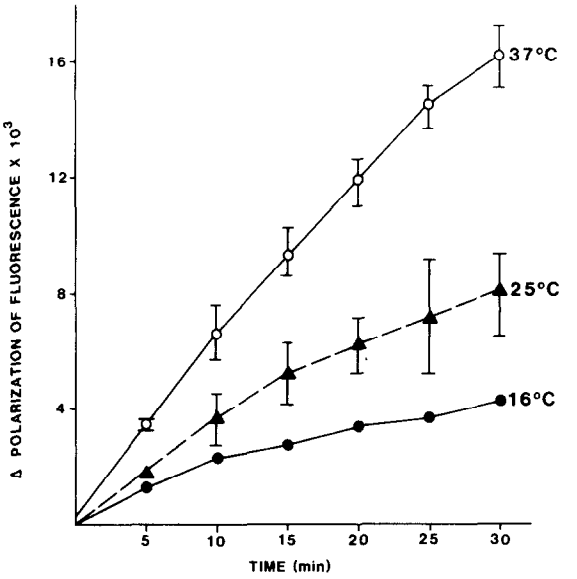


Fig. 1. Polarization of fluorescence of *cis*- and *trans*-parinaric acid incubated with rat brain synaptosomes. Polarization was measured at 37°C under N_2. The cuvette contained: synaptosomes (0.10 mg protein/ml), 5 mM $MgCl_2$ and 1 μM parinaric acid in phosphate-buffered saline. Excitation was at 320 nm with a 370 nm long pass Schot filter in both parallel and perpendicular emission paths to the photomultiplier tubes.

Fig. 2. Effect of temperature on increase of polarization of *cis*-parinaric acid incubated with synaptosomes. Conditions were identical to those in Fig. 1. Temperatures: 37°C, 25°C, 16°C.

TABLE I

POLARIZATION OF FLUORESCENCE OF *CIS*- AND *TRANS*-PARINARIC ACID IN MEMBRANE PREPARATIONS AND PHOSPHOLIPIDS EXTRACTED FROM THE MEMBRANES

All polarization values were measured in an SLM Fluorimeter with the sample under nitrogen, at 25°C and 25 min after addition of 1 μM parinaric acid to a suspension of membranes (100 μg protein/ml) or lipids in phosphate-buffered saline. The lipids used were extracted by the method of Folch et al. [8] from an equivalent amount of membrane preparation and were sonicated for 2 min to obtain an opalesent suspension. Excitation was at 320 nm with a 370 nm long pass Schot filter on both parallel and perpendicular emission paths to the PM tubes. Polarization values were measured in these experiments at 25°C whereas subsequent values were obtained at 37°C. Higher temperatures usually caused a decrease in polarization values. The values are mean values ± S.D. for a single preparation. The S.D. represents precision of the measurement over a data collection period of 2 min with $n = 4$–6. To simplify expression of results the S.D. of the initial values have been deleted, but are of the same order as those of the final values.

Preparation	Polarization of fluorescence			
	Cis-parinaric acid		*Trans*-parinaric acid	
	Initial	Final	Initial	Final
RBC ghosts	0.2523	0.2534 ± 0.0008	0.3136	0.3137 ± 0.0015
Phospholipid extract of ghosts	0.2187	0.2184 ± 0.0007	0.3110	0.3106 ± 0.0003
Brain microsomes	0.2527	0.2660 ± 0.0002	0.3308	0.3313 ± 0.0019
Phospholipid extract of microsomes	0.2089	0.2097 ± 0.0007	0.2978	0.2971 ± 0.0006
Synaptosomes	0.2564	0.2664 ± 0.0006	0.3316	0.3317 ± 0.0003
Phospholipid extract of synaptosomes	0.2189	0.2191 ± 0.0005	0.3178	0.3160 ± 0.0010

and were then incubated with *cis*- and *trans*-parinaric acid (Table I). Both *cis*- and *trans*-parinaric acid in the lipids extracted from each of these membranes, including synaptosomes, have lower polarization values than in the intact membranes. The polarization of fluorescence for *cis*-parinaric acid did not increase when it was incubated with phospholipid extracts for up to 25 min (Table I).

In order to determine if the observed increase in polarization was due to a physical process of lipid bilayer reorganization caused by the addition of exogenous fatty acids or was due to an enzymatic process, the synaptosomes were heated to inactivate the enzymes present. Heating a synaptosomal suspension at 95°C for 5 min caused a 30% inhibition of the polarization increase while heating the suspension to 95°C for 30 min caused a 70% inhibition of the rate of polarization increase when compared to nonheat-treated control samples. This suggested that a relatively heat stable enzymatic process might be involved in the polarization change observed.

Fig. 2 shows the temperature dependence of the increase in polarization of fluorescence of *cis*-parinaric acid incubated with rat brain synaptosomes. The rate of polarization increase at 25°C and 16°C were 50% and 25%, respectively, of the rate at 37°C.

TABLE II

DISTRIBUTION OF FATTY ACID ACYLTRANSFERASE IN RAT BRAIN SUBCELLULAR FRACTIONS

Brain fractions were prepared as described in Methods and Materials. The assay system contained 100 μg protein/ml, 1 μM *cis*-parinaric acid in either 250 mM Tris-HCl, pH 7.4, 0.1 mM CoA, 5 mM Na_2ATP, 5 mM $MgCl_2$, or 250 mM Tris-HCl, pH 7.4 and 5 mM $MgCl_2$. The increase in polarization of synaptosomes after 25 min for synaptosomes with CoA, ATP and $MgCl_2$ was set at 100%. The synaptosomes in the presence of cofactors had an initial polarization value opf 0.2307 ± 0.0011 which increased to 0.2609 ± 0.0009 in 25 min at 37°C with $n = 4$.

Subcellular	Increase in polarization of fluorescence	
	CoA, ATP, Mg^{2+}	Mg^{2+}
Synaptosomes	100	42.2 ± 3.4
Whole brain homogenate	56.6 ± 4.0	36.9 ± 5.0
Mitochondria	62.8 ± 6.1	36.8 ± 5.0
Myelin	32.6 ± 8.2	7.1 ± 3.1
Nuclear	61.9 ± 6.0	13.5 ± 1.1
Microsomes	88.8 ± 6.5	17.5 ± 1.9

Known cofactors required for free fatty acid acyltransferases were tested to determined their effect of the rate of polarization increase (Table II and IV). These will be discussed below, but briefly, $MgCl_2$ caused a stimulation of the polarization rise and chelating any endogenous divalent cations within the synaptosomes with EDTA was more effective in inhibiting (up to 87%) the polarization rise than heating the suspension. Adding ATP plus Mg^{2+} caused a slight stimulation of the polarization rise while further addition of coenzyme A caused a marked stimulation of the process. This provided additional preliminary evidence that the increase in polarization of fluorescence was due to the acylation of *cis*-parinaric acid onto endogenous phospholipids within the membrane.

Identification of reaction products

More convincing proof for the presence and action of a fatty acyltransferase in synaptosomes was sought by the identification of a phospholipid containing covalently attached *cis*-parinaric acid. To this end, larger quantities of synaptosomes were incubated at 37°C with *cis*-parinaric acid in the same ratio as in the polarization experiments. Lipids were extracted and attempts were made to identify the products by TLC. Individual phospholipids were recovered and *cis*-parinaric acid content was estimated from the absorbance of the *cis*-parinaric acid at 319 nm. The polyene conjugated structure of the parinaric acid is easily oxidized so that quantitation and identification by this method gave very erratic results. This was due, in part, to the repeated drying of the organic extract obtained by the Folch procedure and the susceptibility to oxidation of parinaric acid on an activated silica gel plate.

For these reasons, the extraction procedure described by Conner et al. [9] was used since the organic extract is not taken to dryness. High performance liquid chromatography using the procedure described by Chen and Kou [10] was used to avoid the oxidation problems associated with TLC. The absorbance of the lipid extract could be monitored at 203 nm (absorbance by double bonds) to establish the chromatographic profile of the phospholipid classes and a second identical sample

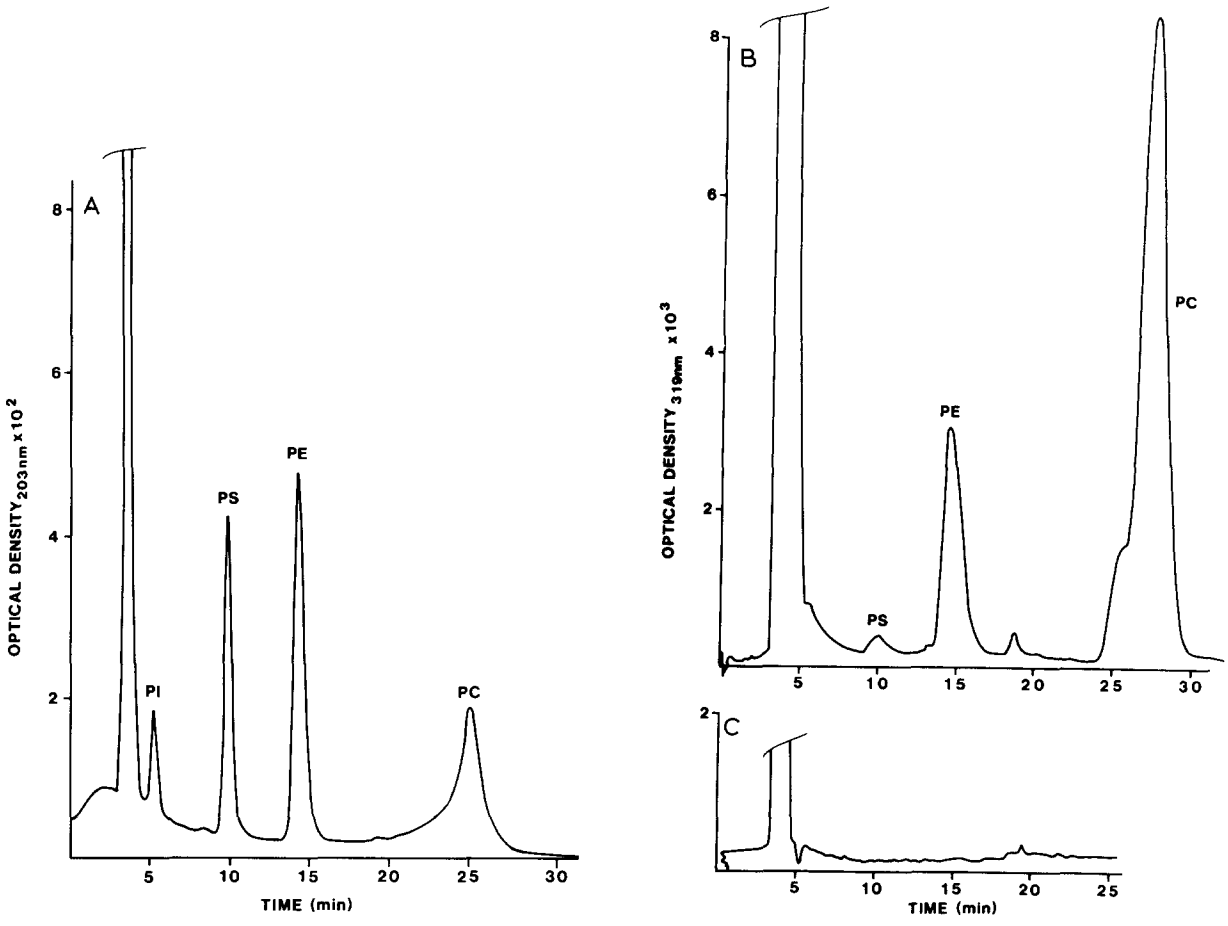


Fig. 3. HPLC analysis of phospholipid extracts of synaptosomes incubated with *cis*-parinaric acid. (A) shows the pattern monitored at 203 nm when 10 μl of extract containing 40 nmol phospholipid phosphorus was injected. B and C shows patterns monitored at 319 nm when 100 μl of extract was injected. In (B) 5 mM $MgCl_2$, 3 mM ATP, were 0.1 mM CoA were supplied while in (C) the synaptosomes were heat inactivated for 45 min at 95°C prior to addition of the substrate *cis*-parinaric acid. Details of procedure are in Materials and Methods.

could be monitored at 319 nm (polyene absorption) where only parinaric acid absorbs light.

Fig. 3(A) presents the resolution by HPLC of the phospholipid classes detected at 203 nm. As shown here, the major phospholipids, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI) are easily resolved. Sphingomyelin elutes well after PC and is barely detectable based on absorbance at 203 nm. Fig. 3(B) shows the distribution of *cis*-parinaric acid in the phospholipid classes based on absorbance at 319 nm. *Cis*-parinaric acid was shown to comigrate with membrane phospholipids in Fig. 3(B) and presumably is covalently attached. It was mainly incorporated into PE (20%) and PC (74%) although traces were found in PI (3%) and PS (2%) (Table III). No incorporation into sphingomyelin was found. The free fatty acid appears at the solvent front in this solvent system. There appears to be greater amounts of *cis*-parinaric acid incorporated into PC relative to the total amount of this phospholipid present in the membrane as shown in Table III. Fig. 3(C) shows the HPLC profile for the identical quantity of extracted phospholipids as studied in Fig. 3(B) except the enzyme was heat inactivated prior to the addition of the fluorescent fatty acid. No phospholipids labeled by *cis*-parinaric acid

TABLE III

DISTRIBUTION OF *CIS*-PARINARIC IN INDIVIDUAL PHOSPHOLIPIDS FROM SYNAPTOSOMES

Synaptosomes were incubated with 1 μM [^{3}H]oleic acid or 1 μM *cis*-parinaric acid in 0.1 mM CoA, 3 mM ATP, and 5 mM $MgCl_2$ in 250 mM Tris-HCl buffer, pH 7.4 for 2 h as described in Methods. Phospholipids were extracted as described by Connor et al. [9]. Extracts were separated on a Waters μPorasil (silica) column with acetonitrile/methanol/85% H_3PO_4 (130:5:1.5, v/v). Areas from the absorbance scans at 203 nm (total phospholipid) and 319 nm (*cis*-parinaric acid) were integrated and the distribution calculated from those values. When [^{3}H]oleic acid was used as substrate column effluent was monitored at 203 μm and fractions of phospholipids were collected and counted. In the experiment shown 8.4% of [^{3}H]oleic acid was incorporated into the total phospholipids. SM, sphingomyelin; n.d., not detected.

Phospholipid class	% total phospholipid (as 203 nm absorbing material)	% total [a] phospholipids	% total *cis*-parinaric (319 nm absorbing material)	% [^{3}H]oleic acid
PI	5.7	3.5	4.7	2.7
PS	19.3	12.6	1.6	1.5
PE	35.5	37.5	20.0	17.2
PC	39.4	37.2	73.5	78.4
SM	n.d.	5.1	0	0

[a] Data from Refs. 11 and 12.

were detected in this extract and all the fluorescent fatty acid was eluted at the solvent front.

Radioactive oleic acid was used as substrate for the transferase reaction with synaptosomes and identical conditions used with cis-parinaric acid. The pattern of distribution of [^{3}H]-oleic acid into the phospholipid classes was almost identical to that seen with *cis*-parinaric acid (Table III). PC was acylated to the greatest extent (74%) while PE had less (20%) and only traces were found in PS or PI.

Sphingomyelin was difficult to detect by its extinction at 203 nm probably due to a low degree of unsaturation in its single fatty acid. Rat brain membranes contain approx. 5–6% sphingomyelin [11,12] (Table III) but neither [^{3}H]oleic acid nor *cis*-parinaric acid were found in the position where authentic sphingomyelin elutes from the HPLC column.

Characteristics of the acyltransferase system

In initial studies, several brain subcellular fractions were studied, and synaptosomes elicited the greatest change in polarization of fluorescence with time after addition of *cis*-parinaric acid. Freshly prepared synaptosomes showed less change in polarization than frozen preparations (data not shown). Treatments that rupture the membrane, e.g., sonication, freezing and thawing, and osmotic shock (data not shown) all stimulated the rate of increase of polarization.

Assigning a value of 100% to the polarization change of *cis*-parinaric in synaptosomes suspended in phosphate-buffered saline the polarization change increased 254% when the membrane preparation was sonicated 2 min in a Branson 200 Sonifier. This effect could be due to increasing the accessibility of the enzyme within the membrane to the added *cis*-parinaric acid, or to releasing essential cofactors from subcellular compartments, or both. Without sonication the reaction could be stimulated to 329% by the addition of 5 mM $MgCl_2$, 0.10 mM CoA and 5 mM ATP. When the preparation has been supplemented with these cofactors sonication caused a stimulation of 382%. This was a much smaller increase than that found in phosphate-buffered saline. Considerable differences in the polarization change were found with different synaptosomal preparations if the reaction medium was not supplemented with coenzyme A and ATP. Using intact synaptosomes in phosphate-buffered saline without cofactors added, the variation in increase in polarization after 25 min was from 4 to 24% with different prepara-

tions (data not shown). Generally, addition of CoA plus ATP stimulated the polarization change from 42 to 230% (Table IV) depending on the preparation used.

The acyltransferase was present in all the brain subcellular membrane fractions examined. Synaptosomes in the presence of exogenous CoA and ATP, had the greatest acyltransferase specific activity of the membranes examined (Table II). The microsomal fraction was only slightly less active than synaptosomes and the nuclear and mitochondrial fractions had similar activities (about 60% of the synaptosomal fraction) while myelin had the lowest activity of fractions surveyed (33% of synaptosomal fraction).

The effects of exogenous cofactors on these subcellular fractions are shown in Table II. Here membrane fractions had relatively low acyltransferase activity in the absence of added cofactors, while subcellular fractions containing enclosed structures (e.g., synaptosomes and mitochondria) were less dependent on Mg^{2+}, CoA and ATP.

Cofactors that have been reported to have an effect on the acyltransferase were tested (Table IV) individually and in combination, for their action on the change in polarization of *cis*-parinaric acid incubated with synaptosomes. Nucleotides and EDTA were generally inhibitory (Table IV, assayed in phosphate-buffered saline) probably due

TABLE IV

REAGENTS AFFECTING RATE OF POLARIZATION CHANGE OF *CIS*-PARINARIC ACID IN SYNAPTOSOMES

In the first experiment, synaptosomes (100 μg protein/ml) in phosphate-buffered saline were preincubated in the indicated medium at 37°C for 10 min and 1 μM *cis*-parinaric acid was added and the increase in polarization was measured at 37°C after 25 min. The data shown in this representative experiment are from a single preparation that presumably contained some endogenous Mg^{2+} and CoA. The initial polarization of fluorescence was 0.2302 ± 0.0009. In the second experiment in Tris-HCl buffer, CoA and ATP were initially present during the 10 min preincubation. Additions were made as before and polarizations were measured after 25 min. The initial polarization of fluorescence was 0.2342 ± 0.0008.

Medium	Additions	Polarization change (%)
Phosphate-buffered saline	None	100
	5 mM $MgCl_2$	111.7 ± 7
	5 mM $CaCl_2$	116.8 ± 8
	5 mM $MgCl_2$; 3 mM ATP	135.2 ± 3
	0.1 mM Coenzyme A	105.2 ± 3
	5 mM $MgCl_2$; 0.1 mM CoA	131.1 ± 7
	5 mM $MgCl_2$; 3 mM ATP; 0.1 mM CoA	140.5 ± 9
	3 mM ATP	89.7
	1 mM AMP	88.4
	5 mM $MgCl_2$; 3 mM CTP	66.8
	5 mM β-mercaptoethanol	101.2 ± 7
	125 mM EDTA	52.3 ± 7
	washed and resuspended in 125 mM EDTA	12.9
250 mM Tris-HCl, pH 7.4; 0.1 mM CoA, 3 mM ATP	5 mM $MgCl_2$	100
	5 mM $MgCl_2$; 2.5 μM lysoPC	116
	5 mM $MgCl_2$; 5.0 μM lysoPC	130.2
	5 mM $MgCl_2$; 50.0 μM lysoPC	131.5
	5 mM $MgCl_2$; 10 mM NaF	39.2
	5 mM $MgCl_2$; 50 mM NaF	45.5
	5 mM $MgCl_2$; 5 μM oleic acid	58
	5 mM $MgCl_2$; 20 μM oleic acid	39.1
	5 mM $CaCl_2$	151.1 ± 4
	5 mM $MgCl_2$; 5 μM $CaCl_2$	16.1
	2.5 mM $MgCl_2$; 2.5 μM $CaCl_2$	27.6

to chelation of divalent cations. Mg^{2+} and Ca^{2+} were stimulatory but the highest stimulation was routinely observed in the presence of Mg^{2+}, CoA and ATP. CoA alone or β-mercaptoethanol had little effect.

Since lysophospholipids in the membrane are acceptor molecules for the acyl group, we added 2.5–5.0 μM exogenous lysophosphatidylcholine and found that it stimulated the reaction 16–30%. Synaptosomal membranes treated briefly with phospholipase A_2 from *Crotalus adamanteus* and washed (data not shown) also show an increase in polarization change when compared to untreated samples. NaF is an inhibitor of phospholipase A_2 and other enzymatic reactions. The addition of NaF inhibited the rate of polarization change with *cis*-parinaric acid by 55–60%. Whether this was due to decreased levels of lysophospholipids within the membrane or to some other undefined mechanism is unknown.

Addition of oleic acid inhibited the rate of polarization change (Table IV). This is likely due to the dilution of *cis*-parinaric acid in the free fatty acid pool within the membranes.

It was found that calcium was a more effective divalent cation in this system than magnesium in promoting acyltransferase activity. When both cations, Ca^{2+} and Mg^{2+} were present, a marked inhibition of the reaction was found. This accounts for earlier, rather erratic, results found upon addition of calcium. This inhibition was found even when the sum of the two cation concentrations was 5 mM the normal concentration used with either cation used individually.

Polarization of parinoyl-phospholipids in synaptosomes

Quantitation of the amount of product formed is essential if the current work is to be used for routinely assaying acyltransferase activity. The amount incorporated could be validated if radioactively-labeled *cis*-parinaric acid were available. However, with [^{3}H]oleic acid we found that 8.4% of the available substrate was incorporated into total synaptosomal phospholipids in 60 min at 37°C. Since oleic acid is not fluorescent no determination of polarization changes was possible.

An indirect approach has been pursued in the case of *cis*-parinaric acid. An attempt was made to estimate the polarization value of the acylated phospholipid within the membrane from the change in polarization after incorporation of *cis*-parinaric acid and the corresponding increase in fluorescence intensity (Table V). We assumed two pools of fatty acids: free and incorporated into some phospholipid. It was assumed that all the *cis*-parinaric acid contributing to fluorescence at 1 min (values F_0 and P_0, Table V) was in the free

TABLE V

POLARIZATION OF FLUORESCENCE OF ACYLATED PHOSPHOLIPID WITHIN SYNAPTOSOME MEMBRANES

Experiments a, b, and c show data on three separate synaptosomal preparations and the change in fluorescence intensity and polarization after 25 min at 37°C. The final column contains the calculated values for the polarization of fluorescence of the acylated phospholipid using the relationship:

$$(F_0P_0)+(F_f-F_0)P_{pl}=F_fP_f$$

where F_0 = initial relative fluorescence, P_0 = initial polarization of fluorescence, F_f = final relative fluorescence, P_f = final polarization of fluorescence, P_{pl} = polarization of fluorescence of acylated phospholipid.

Experiment	F_0 (Initial intensity)	P_0 (Initial)	F_f (Final intensity	P_f (Final)	P_{pl}
a	5690	0.2272	6345	0.2433	0.383
b	6546	0.2299	7662	0.2532	0.389
c	5588	0.2030	6054	0.2176	0.391
16 h incubation			3141	0.2909	–
16 h, bovine serum albumin washed	–	–	955	0.3847	–

acid form somewhere within the lipid bilayer. Secondly, the increases in polarization (P_f) and fluorescence intensity (F_f) were due only to the acylation reaction. The unreacted *cis*-parinaric acid was in a pool in the bilayer and had a constant polarization of fluorescence. With these assumptions, a polarization of acylated phospholipids between 0.36 and 0.39 was calculated (Table V). This value is within a reasonable range for a molecule the size of the phospholipid within a lipid bilayer.

A second more direct method was used to estimate the polarization value of the acylated phospholipid. Albumin is known to have very high affinity binding sites for free fatty acids. *Cis*-parinaric acid was incorporated into synaptosomal membrane phospholipids and then attempts were made to remove the free fatty acid from the bilayer by incubating with bovine serum albumin. This treatment should leave the acylated phospholipids of *cis*-parinaric acid in the bilayer for direct measurement of the polarization value. A polarization value of 0.29 was obtained by incubating synaptosomes under nitrogen with $MgCl_2$, CoA and ATP for 16 h (Table V). The initial polarization value was 0.22. This sample was incubated with 5 mg bovine serum albumin/ml in phosphate-buffered saline, and then the synaptosomes were washed free of bovine serum albumin. The value of the polarization of the resulting material (Table V) was 0.38 which is the same as the calculated value from the earlier experiments. Based on the fluorescence intensity of the bovine serum albumin-treated synaptosomes we estimate that about one-third of the added *cis*-parinaric acid was incorporated into the membrane phospholipids. After 25 min incorporations of *cis*-parinaric acid, about 5–10% of the added probe was incorporated into membrane phospholipids. This is based on the data in Table V for the estimated polarization of the acylated product (0.36) and the value for nonacylated *cis*-parinaric acid within the membrane (0.21). Under the reaction conditions used (2 nmol total *cis*-parinaric acid in 2 ml, containing approx. 200 μg synaptosomal protein) we estimate that 1.5–4 nmol *cis*-parinaric acid was incorporated per mg protein per h at 37°C.

Discussion

In this report we propose that the increase in polarization of fluorescence that occurs when the fluorescent fatty acid *cis*-parinaric acid is incubated with synaptosomes or other brain membranes is the result of the action of a fatty acyltransferase within the membrane. 2-Acyltransferase has been reported to preferentially utilize unsaturated, *cis*, C16–C20 fatty acids [13] like *cis*-parinaric acid and may be active in the preparations that we have studied here. The acceptor molecules within membranes for the parinaric acid are presumably lysophospholipids generated by endogenous phospholipases. NaF, a known inhibitor of phospholipases, inhibited incorporation of *cis*-parinaric acid, supporting the role of lysophospholipids in the reaction studied in this paper.

In this study we attempted to define the cause of the increase in fluorescence polarization when *cis*-parinaric acid was added to brain synaptosomes. It appears that an enzymatic process rather than a physical incorporation into the lipid bilayer is involved since the increase in polarization was: (a) inhibited by heat treatment, (b) temperature dependent, (c) not found in all membranes, (d) not found in lipid extracts of membranes and (e) stimulated by specific cofactors.

Acyltransferase activity was found in all the brain membrane fractions examined and this is not unexpected since the acyltransferase is directly involved in phospholipid metabolism. The synaptosomal fraction had a slightly higher activity than the microsomal fraction and in the absence of added cofactors, microsomes were much less active than synaptosomes. Those membranes existing as enclosed structures, i.e., synaptosomes and mitochondria, generally had the greatest activity without the addition of cofactors. In the presence of the most important cofactors, i.e., CoA, ATP and Mg^{2+} or Ca^{2+}, these differences were not as great (Tables II and IV). Any treatment that disrupted and fractured the membranes, e.g., freezing and thawing, osmotoc shock, or sonication, increased the reaction rate for incorporation of fatty acid by making the enzyme and substrate more accessible to each other and perhaps by releasing cofactors from their membrane-bound compart-

ments. Individually, the cofactors have a small stimulatory effect on the rate of polarization increase and this likely depends upon other limiting concentrations of endogenous cofactors. Chelation of divalent cations by EDTA was one of the most effective methods of inhibiting the reaction.

When required cofactors were supplied the interaction between free fatty acid and the acceptor lysophospholipid could be examined. Adding exogenous lysophosphatidylcholine (2.5–5 μM) stimulated the change in polarization rate by 16–30% probably showing that additional acceptor will stimulate the reaction. It is not certain at present whether this really represents increased acceptor concentration in the assay or an indirect detergent-like action of the lysophospholipid.

The addition of oleic acid caused a decrease in the rate of polarization change, most likely by diluting the proportion of the fluorescent fatty acid pool within the membrane. Oleic acid is an acceptable substrate for the acyl transferase as shown in Table III. NaF probably decreased the acylation reaction by inhibiting the phospholipase A_2 reaction and thereby decreasing the amount and availability of lysophospholipid acceptor. The greater stimulation by Ca^{2+} over Mg^{2+} may be due to stimulation of the phospholipase A_2 reaction by Ca^{2+} so that more lysoPC acceptor is available as well as due to its action as a divalent cation cofactor in the acyltransferase reaction. When both Ca^{2+} and Mg^{2+} were added together strong inhibition (72%) of the rate of polarization change was found even when the total concentration of divalent cation was 5 mM. Whether this is a feedback control mechanism or an allosteric binding effect is not known, but this interaction is being investigated.

Although phosphatidylcholine represented only 37% of all phospholipids, 74% of the incorporated **cis**-parinaric acid appeared here (Table III, Fig. 3B). One could infer from the data that the acyltransferase has a preference for lysophosphatidylcholine as acceptor of the *cis*-parinaric acid. However, this is not necessarily the case. Alternatively the concentrations of other lysophospholipids may be significantly lower within the membrane. Aside from enzyme specificity it is possible that the *cis*-parinaric acid partitions into a phosphatidylcholine enriched portion of the lipid region of the membrane binding the acyltransferase thus leading to increased incorporation.

The rate of increase in polarization of fluorescence increased when the concentration of *cis*-parinaric acid was increased from 1 to 7 μM. Further increases in concentration of *cis*-parinaric acid became slightly inhibitory. Also the initial polarization value decreased with increasing free fatty acid concentration both with *cis*-parinaric acid, but also with addition of oleic acid. This would indicate that the free fatty acid substrate itself had a fluidizing effect on the membrane.

The comparison of the polarization of fluorescence of both isomers of parinaric acid in biological membranes and the phospholipids extracted from those membranes provides information on the effect membrane proteins exert on the lipid regions of the membranes. In all six cases examined (Table I), the polarization of fluorescence of the fatty acid was greater within the membrane than in the extracted lipids. This would indicate that a more rigid, less fluid environment exists when proteins are present. It is evident that the two isomers must be located in different regions of the lipid domain within the membrane as suggested by Sklar et al. [4]. *Cis*-parinaric acid showed a much larger change in polarization than *trans*-parinaric acid when present in membranes instead of lipid extracts. This could mean that the *cis*-parinaric acid segregates near proteins in the membrane while the *trans*-parinaric acid tends more to be associated only with other lipids in the bilayer. In red blood cell ghosts differences in polarzation of *trans*-parinaric acid were less evident (Table I), however, acyltransferase activity was not found in red cell ghosts.

The polarization of fluorescence of the parinoyl phospholipid(s) in Table V were higher than expected. This could have been due to several causes. (1) During the isolation procedure, myelin membrane fragments could have contaminated the synaptosomal fraction. Myelin has been shown to cause a higher polarization of fluorescent probes than synaptosomes. (2) The large polyunsaturated fatty acid content usually found in synaptosomes could have been oxidized, which would result in a high polarization measurememt. This seems unlikely. (3) The most probable cause is that *cis*-parinoyl phospholipids reside in a highly ordered

lipid environment near or bound to proteins in the membrane. This would seem more probable due to *cis*-parinaric acids partitioning into both fluid and gel-like regions of membranes [14].

Using the change in polarization of *cis*-parinaric acid as an indirect method for monitoring the activity is a free fatty acid acyltransferase has advantages and disadvantages. A major advantage is that using the fluorescent probe, reaction rates can be determined within 30 min. The classical procedure requires at least a day's work and involves several separate steps: (1) the incubation period, (2) extraction, (3) thin-layer chromatography, and (4) scraping and counting material from the plates. The present procedure is therefore much faster. Also, in the fluorescent procedures using 150 μl microcells for the fluorometer, as little as 15 μg of protein was required to assay enzyme activity. Generally then, sensitivity also is greater than in the classical procedure. Also, continuous kinetic analysis is possible with one sample by observing changes in polarization. With the classical method, each time point requires a separate sample and analytical procedure.

There are three disadvantages to this fluorescent procedure. First, a fluorescent fatty acid not normally present in mammalian membranes is used as a substrate. This raises the question of the applicability of data obtained with this substrate to the metabolism of normal mammalian fatty acids. This is of concern whenever any synthetic substrate is used to monitor an enzyme reaction, but the almost identical distributions of [^{3}H]oleic acid and *cis*-parinaric acid in membrane phospholipids after action of the acyltransferase supports the use of this fluorescent probe. Second, the conjugated structure of parinaric acid is very labile to oxidation which would influence the fluorescent data should oxidation occur during the reaction. Third, a major limitation to the procedure is quantitation of the reaction product, *cis*-parinoyl-phospholipid, by an indirect physical parameter. A close approximation of the amount of product formed may be calculated from the change in polarization. The assumption that the polarization of fluorescence will be identical for all phospholipids acylated within the membrane may not be justified. On balance it is felt that the advantages of this method outweigh the disadvatages for certain studies with membrane preparations.

Our calculated value for the rate of the acyltransferase reaction is much lower than the value reported by Wise et al. [2], who used rat brain microsomes. Wise et al. [2] found that 1.2–3 nmol fatty acid per mg protein per min was incorporated using oleoyl-CoA as substrate with 10 to 100 nmol of exogenous added lysoPC. Using oleic acid as substrate, and CoA, these authors reported rates half as great as when oleoyl-CoA was used. Without added lysoPC, their values were one-tenth those with added lysoPC. The rates estimated in this report, using free fatty acid (*cis*-parinaric acid) and CoA without exogenous lysophospholipid, are similar to those in the report of Wise et al. [2] under similar conditions but are only 5% of their maximal values.

The advantages of being able to modify rapidly the fluidity of a biological membrane are obvious. With the acyltransferase activity, it would be possible to change the fatty acid composition of phospholipids within the membrane without having to translocate the entire phospholipid molecule [15]. The 2-acyl position is reported to be that portion of the phospholipid molecule that confers the fluidity character to the phospholipid [13] and this position preferentially has a high percentage of unsaturated fatty acids [13]. With modification of the fatty acid composition at the 2-position, a major change in fluidity could be accomplished. This event must also be occurring at limited regions within the lipid domain of the membrane.

The fluorescent procedure presented allows a relatively rapid, continuous assay of an acyltransferase involved in phospholipid metabolism in biological membranes.

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