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PROTEIN-MEDIATED EXCHANGE OF SYNTHETIC PHOSPHATIDYLCHOLINES INTO SYNAPTOSOMAL MEMBRANES

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A phosphatidylcholine (PC) exchange protein from bovine liver was used to exchange endogenous synaptosomal membrane PC's with PC's of defined fatty-acid composition from phospholipid vesicles. Up to 50% of the total synaptosomal PC could be exchanged during a 3 h incubation with PC's which were in the liquid-crystalline state at the temperature of incubation (dimyristoyl-, dioleoyl- and dielaidoyl-PC). The biphasic kinetics of the exchange of ^{14}C-labeled 1-palmitoyl-2-oleoyl-PC into isolated synaptic plasma membrane vesicles indicated that the half-time for transbilayer equilibrium of PC in these membranes was about 10 h. Hence, the observed 50% exchange of total synaptosomal PC probably represented nearly complete exchange of PC in the outer face of the synaptosomal plasma membrane. This extensive exchange was accomplished without apparent loss of synaptosomal function, including membrane potential and high-affinity uptake of choline and γ-aminobutyric acid. PC's in the gel state (dipalmitoyl- and distearoyl-PC) could not be exchanged extensively into the synaptosomal membranes. However, from within gel-state distearoyl-PC liposomes, a trace amount of fluid 1-palmitoyl-2-oleoyl-PC (T_m < 10°C) could be preferentially exchanged into the synaptosomes at 32°C with little transfer of the saturated PC.

Introduction

A number of enzymes associated with membranes require phospholipid or are modulated by phospholipid in their function [1–3]. Lipid-exchange proteins offer a powerful new approach for modifying the lipid composition of biological membranes as a means of studying the influence of lipid environment on the activity of membrane-bound enzymes and carriers [4–8]. Exchange proteins with differing lipid specificities have been purified [9–13] and allow the modification of membrane-lipid composition under mild conditions. Recently, we reported the use of a non-specific lipid transfer protein to alter the cholesterol-to-phospholipid ratio of synaptosomes and synaptic plasma membranes [4]. We observed a specific and reversible loss of γ-aminobutyric-acid uptake with the lowering of membrane cholesterol-to-phospholipid ratio. In the present study, we report the first use of a specific phosphatidylcholine (PC) exchange protein in exchanging the endogenous PC pool of synaptosomal membranes with synthetic PC's of defined fatty-acid composition. The exchange protein was also used to estimate the distribution and translocation

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Abbreviations: PC, phosphatidylcholine; CC_5, 3,3′-dipentyl-2,2′-oxacarbocyanine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Krebs-Hepes medium, 130 mM NaCl/5 mM KCl/1.3 mM $MgCl_2$/1.2 mM sodium phosphate/10 mM glucose/0.5 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid/10 mM Hepes (pH 7.4); T_m, phospholipid liquid-crystalline to gel-state transition temperature.

rate of PC between the outer (exposed) and inner halves of the bilayer of isolated synaptic plasma membrane vesicles. The effect of the changes in PC fatty-acid composition on synaptosomal function, including membrane potential and Na^+-dependent choline and γ-aminobutyric-acid uptake, was analyzed.

Materials and Methods

Lipids

Several types of L-α-phosphatidylcholine were obtained from Sigma Chemical Company (St. Louis, MO): egg-yolk PC (type V-E), dimyristoyl-PC (98%), dipalmitoyl-PC (99%), distearoyl-PC (99%) and dioleoyl-PC (98%). 1,2-Dielaidoyl (18 : 1 *trans*)-*sn*-glycero-3-phosphorylcholine was synthesized and purified in our laboratory by the method of Warner and Benson [14]. Cholesterol (over 99%) was purchased from Eastman Organic Chemicals (Rochester, N.Y.). [2-^{3}H]Glycerol trioleate (triolein), 2 Ci/mmol, was from ICN Pharmaceuticals (Irvine, CA). The triolein was purified by thin-layer chromatography on Silica Gel H with hexane/diethyl ether/acetic acid (70 : 30 : 1, v/v) and stored in hexane at −20°C. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoryl[*N-methyl*-^{14}C]choline (0.8 mCi/mmol) was synthesized and purified by Dr. Hans Eibl in our laboratory [15].

Egg-PC phospholipid vesicles (liposomes) containing trace amounts of [^{14}C]PC and [^{3}H]triolein (6000 and 60000 dpm/μg P, respectively) were prepared for use in the assay of exchange protein activity. The lipids (1–2 mg of lipid) were mixed in chloroform in acid-cleaned glass-stoppered tubes and dried to form a thin film under a stream of N_2. The lipids were dispersed in 0.25 M sucrose/50 mM Hepes/1 mM EDTA (pH 7.4) at 1 mg PC/ml and sonicated to clarity (10–20 min) in a bath sonicator under nitrogen atmosphere.

For use in phospholipid replacement experiments, PC's of selected type with or without 0.5 mol of cholesterol/mol of PC were mixed in chloroform with a trace of [^{3}H]triolein (and sometimes ^{14}C-labeled PC) in small round-bottomed flasks. After evaporation of the solvent under a stream of N_2, the lipids were dried further under vacuum in a desiccator preflushed with N_2. The lipids were then dispersed in 0.25 M sucrose/50 mM Hepes (pH 7.4) at 5–12 mg PC/ml by a brief partial submersion of the flasks in the bath sonicator. After hydration in the cold for at least 1 h, egg-PC and dioleoyl-PC liposomes were sonicated in glass tubes, under nitrogen, until translucent (20–30 min) in the bath sonicator at 25–35°C. Distearoyl-PC liposomes and all liposomes containing cholesterol were sonicated at ambient temperature using a Branson sonicator with a $\frac{1}{2}$-inch tipped horn, 90% duty cycle, under a gentle stream of N_2. Three 4-min periods of such sonication, alternated with cooling periods at room temperature of equal duration, were sufficient for production of translucent dispersions. Dielaidoyl-PC and dimyristoyl-PC liposomes were similarly sonicated with the probe at 50% and 70% duty cycle, respectively, for two 4-min periods. Liposomes dispersed with the probe were centrifuged at $20000 \times g_{av}$ for 45 min at 25°C to remove titanium fragments and undispersed lipid. Liposomes were stored at room temperature and were used no more than 4 h after sonication.

Other isotopes and chemicals

γ-Amino[U-^{14}C]butyric acid (224 mCi/mmol) was obtained from Amersham (Arlington, IL), and [*methyl*-^{3}H]choline chloride (80 Ci/mmol) was from New England Nuclear (Boston, MA). Choline bromide and γ-aminobutyric acid were obtained from Sigma Chemical Company (St. Louis, MO). The CC_5 was a gift of Dr. Alan Waggoner (Department of Chemistry, Amherst College, Amherst, MA).

Protein and lipid analysis

Protein was determined according to Lowry et al. [16] or, when mercaptoethanol was present, by the modification described by Ross and Schatz [17]. Bovine serum albumin was used as a standard. Phosphorus was measured by the method of Chen [18] as described in Rouser and Fleischer [19].

Extraction of membrane lipids was carried out according to Bligh and Dyer [20] under a N_2 atmosphere and protected from light. Recovery of [^{14}C]PC during extraction was greater than 95%. Phospholipids were separated by one-dimensional thin-layer chromatography on silica gel H in a solvent system consisting of either chloroform/

methanol/water (65 : 25 : 4, v/v) or chloroform/methanol/acetic acid/water (50 : 25 : 6 : 3, v/v). Precoated plates of 0.25 mm gel thickness (Analtech, Newark, DL) were activated by heating at 120°C for 20 min. The chromatographic solvents were bubbled with N_2 before use when phospholipids were to be separated for subsequent fatty-acid analysis. Approx. 10 μg of lipid phosphorus was applied to each plate. For determination of phospholipid composition, the lipid bands were revealed by charring for 30 min at 180°C after spraying with H_2SO_4/30% formaldehyde (97 : 3, v/v), then scraped for phosphorus analysis. The identity of each band was determined by comparison to the relative migration of synthetic phospholipid species or a well-defined mitochondrial phospholipid fraction. For fatty-acid analysis, the lipid bands were visualized by spraying with a water mist. The plates were then dried under N_2, and the marked bands were scraped into 13 ml glass-stoppered tubes and dried for 1 h in a vacuum desiccator, preflushed with N_2. The samples were incubated with 1.5 ml of 14% BF_3 in methanol (Applied Science, Station College, PA) for 5 min at 65–70°C in an oil bath. The methyl esters were extracted with 1 ml of *n*-hexane and 1.5 ml of deionized water by briefly vortexing. After a brief centrifugation (1 min), the upper (hexane) phase was set aside. The water phase was extracted twice more with 1 ml hexane, and the combined hexane phases were dried under a stream of nitrogen to about 0.05 ml for gas-liquid chromatography. The transesterified methyl esters were analyzed in a Barber-Colman series 5000 Gas Chromatograph using 6-foot columns containing 3% OV 225 or 10% SP 2330 on 100/120 Chromatosorb W AW (Supelco, Bellefonte, PA). The column temperature was 200°C. The methyl ester peaks, detected by flame ionization, were identified by relative retention times and by comparison to standard mixtures (Applied Science). The relative content of fatty acids in the samples was quantitated using the peak height × retention time method [21].

Preparation of synaptosomes and synaptic plasma membranes

Rat forebrain synaptosomes were prepared and suspended in Krebs-Hepes medium containing 0.2 M sucrose as previously described [4]. The synaptosomes were used directly after preparation for lipid exchange.

Synaptic plasma membranes were prepared from rat forebrain as described by Jones and Matus [22]. The final membrane pellet was suspended in 0.32 M sucrose/2.5 mM Hepes (pH 7.4) to 4–10 mg protein/ml, then rapidly frozen in liquid N_2 and stored at −85°C.

Measurement of synaptosomal functions

Membrane potential. The synaptosomal membrane potential was estimated using the permeant fluorescent cation 3,3′-dipentyl-2,2′-oxacarbocyanine (CC_5) as described previously [4]. As an index of membrane potential, $\Delta F_{65\ mM\ K^+}/\Delta F_{5\ mM\ K^+}$ was calculated, where $\Delta F_{5\ mM\ K^+}$ and $\Delta F_{65\ mM\ K^+}$ are the changes in fluorescence upon addition of the synaptosomes to the dye in 5 mM K^+ medium and upon further addition of 65 mM KCl, respectively.

Uptake of choline and γ-aminobutyric acid. Na^+-dependent uptake of γ-amino[^{14}C]butyric acid and [^{3}H]choline by synaptosomes was measured in a single assay mixture by a centrifugation method described by Simon and Kuhar [23] for [^{3}H]choline. The incubation medium was Krebs-Hepes (pH 7.4) containing 0.2 M sucrose, 1 μM γ-amino[U-^{14}C]butyric acid and 1 μM [*methyl*-^{3}H]choline chloride (37°C). Sample blanks without radioactive γ-aminobutyric acid and choline were used to subtract the small contribution (less than 10%) of ^{14}C- and ^{3}H-labeled lipids to the total radioactivity of the synaptosomal pellets.

Preparation and assay of the PC exchange protein

The PC exchange protein was partially purified from a pH 5.1 supernatant of beef liver by pH 3 precipitation followed by DEAE-cellulose and CM-cellulose chromatography essentially as described by Kamp et al. [9]. The Sephadex G-50 chromatography was omitted. The active fraction eluted from the DEAE-cellulose was concentrated to 200 ml and exchanged with 1 liter of 10 mM citric acid/20 mM sodium phosphate/10 mM β-mercaptoethanol (pH 5.0) using an Amicon hollow-fiber filtration apparatus in preparation for CM-cellulose chromatography. The active fraction

eluted from the CM-cellulose column was stored at −20°C in a 1:1 mixture of elution buffer and glycerol. Before use of the exchange protein, the glycerol was removed by dialysis against either 5 mM sodium phosphate/10 mM β-mercaptoethanol (pH 7.4) or 0.32 M sucrose/5 mM β-mercaptoethanol/25 mM Hepes (pH 7.4). In some cases the dialyzed exchange protein was concentrated 2–3-fold in an Amicon ultrafiltration cell with a PM-10 filter under N_2 pressure. There was little or no loss of activity during dialysis or concentration.

Exchange activity was assayed by following the transfer of ^{14}C-labeled PC from liposomes to an excess of heat-treated mitochondria essentially as described by Crain and Zilversmit [13] with the following modifications: egg-PC liposomes (4 μg P) containing 9.0 mol% (0.01 μCi) of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoryl[*N-methyl*-^{14}C]-choline and 0.04 mol% (0.1 μCi) of [2-^{3}H]triolein (ICN, 2 Ci/mmol) were used as the donor liposomes, and the mitochondria (70 μg P) were reisolated for radioactivity measurement following the exchange by centrifugation through a sucrose density layer. The non-exchangeable [^{3}H]triolein marker allowed correction for cosedimentation of liposomes. The specific activity of the exchange protein used in these studies, in units of nanomoles of [^{14}C]PC transferred to the mitochondria/min at 32°C, was about 356 units/mg protein. This represents a 500-fold purification over the pH 5.1 supernatant.

Lipid exchange procedures

Synaptosomes. Synaptosomes were incubated with the exchange protein and liposomes either in Krebs-Hepes medium (pH 7.4) containing 0.2 M sucrose or in 0.32 M sucrose/25 mM Hepes (pH 7.4) supplemented with 10 mM glucose. The synaptosomes were added to the exchange protein in the desired medium and preincubated, usually at 32°C, for 10 min before addition of the liposomes to start the exchange. The final membrane protein concentration was about 1.5 mg/ml, and the liposomal PC to synaptosomal PC ratio was generally between 6 and 15. When the incubations were to exceed 3 h, streptomycin sulfate (0.1 mg/ml) and penicillin (100 units/ml) were included. The buffer composition was invariant for all samples in a given set of exchanges. When the exchanges of different types of liposomal PC were compared, all samples were run concurrently under the same conditions with molar ratios of liposomal to synaptosomal PC as nearly identical as possible. The exchange was terminated by centrifugation of the synaptosomes at 2°C through a layer of 0.5 M sucrose at $80000 \times g_{av}$ for 60 min, or when study of function was to follow, simply at 10000 rpm ($7800 \times g_{av}$) for 10 min in a Beckman JA-20 rotor. The pellets were surface-rinsed with cold 0.32 M sucrose and then resuspended to 2–4 mg of protein/ml in 0.32 M sucrose or, when synaptosomal functions were to be measured, in Krebs-Hepes medium (pH 7.4) containing 0.2 M sucrose. Choline and γ-aminobutyric-acid uptake and membrane potential were assayed without delay.

The extent of exchange of synaptosomal PC with liposomal PC was assayed either directly by fatty-acid analysis or by using ^{14}C-labeled PC. In the latter case, those counts of ^{14}C in the membrane pellet which were referable to contaminating liposomes, obtained by multiplying the counts of [^{3}H]triolein in the pellet by the ^{14}C/^{3}H ratio of the starting liposomes, were subtracted from the total counts of ^{14}C in the pellet to give the quantity of ^{14}C transferred to the membranes by exchange. The latter then was converted to a molar quantity using the measured specific activity of [^{14}C]PC in the starting liposomes (dpm/nmol PC). The percentage exchange of membrane PC with liposomal PC was defined as the molar percentage of the total membrane PC which was ^{14}C-labeled. The quantity of membrane PC in each sample was estimated using the measured phosphorus content of the sample (corrected for liposome contamination as described above) and the known fraction of synaptosomal lipid phosphorus referable to PC (0.4). When PC exchange was quantitated by performing fatty acid analysis on the exchanged membranes, liposome sticking was corrected for either on the basis of the non-exchangeable [^{3}H]triolein marker as described above or on the basis of the apparent increase in the PC pool size (ratio of PC to total phospholipid, based on phosphorus) of the membrane. The latter was determined by thin-layer chromatography followed by phosphorus analysis on the separated phospholipid species. Since the PC exchange process

itself does not significantly alter phospholipid composition in the donor and acceptor membranes [24], any observed increase in the PC pool size was entirely due to the presence of contaminating PC liposomes which were of known fatty-acid composition. The apparent PC fatty-acid composition of the membranes could thus be adjusted for the presence of the liposomes, and the percentage exchange of membrane PC with liposomal PC calculated. Comparable results were obtained using the two methods of correction for liposome sticking.

Synaptic plasma membranes. Exchange of ^{14}C-labeled PC from liposomes into isolated synaptic plasma membrane vesicles was studied as a function of time in order to estimate the rate of equilibration of PC between inner and outer halves of the membrane bilayer. The plasma membranes (0.45 mg protein/ml) were incubated in 0.32 M sucrose/50 mM Hepes (pH 7.4) at 37°C with the exchange protein and egg PC liposomes (2.2 μmol PC/ml) containing 5 mol% (0.09 μCi/ml) of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoryl[*methyl*-^{14}C]choline and a trace of [^{3}H]triolein (0.78 μCi/ml). At appropriate time points, reactions were stopped by 5-fold dilution with cold exchange buffer. 2 ml of cold 0.5 M sucrose were layered into the bottom of the tubes to form a lower density step, and the samples were centrifuged at $100000 \times g_{av}$ for 40 min at 2°C. The membrane pellets were suspended in a small volume of deionized water, and aliquots were taken for counting and protein and phosphorus analysis. The counts of [^{14}C]PC in the pellets were corrected for liposome contamination using the [^{3}H]triolein marker as described above, and the fraction of total membrane PC originating from the liposomes (^{14}C-labeled) was calculated. The fraction of initial membrane PC remaining after the exchange (i.e., 1 minus the fraction which was ^{14}C-labeled) was then expressed on a semilogarithmic plot as a function of time. A biexponential rate was observed and was used to derive the percentage of PC on the exposed face of the vesicles and the half-time for equilibration of PC between outer and inner layers of the membrane. This calculation was based on a two-pool model described by Bloj and Zilversmit [25].

Results

The PC exchange protein was used to exchange endogenous synaptosomal membrane PC's with PC's of defined fatty-acid composition from phospholipid vesicles. The extent of exchange was determined by fatty-acid analysis performed on the synaptosomal membrane PC pool, either from the synaptosome as a whole (Table I) or from the synaptosomal plasma membrane, isolated subsequent to exchange of the synaptosomes with phospholipids (Table II). Up to 50% of the membrane PC could be replaced with liposomal PC's which were in the liquid-crystalline state at the temperature of incubation (dimyristoyl-, dioleoyl- and dielaidoyl-PC). PC's in the gel state at the temperature of incubation (dipalmitoyl- and distearoyl-PC) were exchanged to only a limited extent into the synaptosomal membranes. The PC exchange protein did not catalyze transfer of phosphatidyl[^{14}C]ethanolamine or [^{14}C]cholesterol from liposomes to synaptosomes, nor did it alter the PC pool size or phospholipid/protein ratio of synaptosomes incubated with a 10-fold excess of liquid-crystalline or gel-state PC liposomes (not shown). Fatty-acid analysis confirmed that the fatty-acid composition of phosphatidylethanolamine, which represents about 37% of the synaptosomal phospholipid, was not altered by the exchange procedure (data not shown).

The exchange protein was not random in its selection of individual PC molecules from donor liposomes containing more than one species of PC. The ^{14}C-labeled 1-palmitoyl-2-oleoyl-PC tracer ($T_m < 10$°C; cf. Ref. 26) was exchanged readily into synaptosomes from gel-state distearoyl-PC liposomes without comparable transfer of the saturated liposomal PC (Table II). However, exchange of this marker was relatively representative of bulk PC exchange from liquid-crystalline dioleoyl- and dielaidoyl-PC liposomes (Table II).

Exchange of [^{14}C]PC from egg PC liposomes into synaptosomes during relatively short incubation times (40 min) was decreased by inclusion of cholesterol in the donor liposomes or by salt (Krebs-Hepes) in the exchange medium (Table III). The slower exchange in the presence of Krebs-Hepes medium probably reflects inhibition by cations, especially Mg^{2+} [27]. The slower ex-

TABLE I

THE FATTY-ACID COMPOSITION OF PHOSPHATIDYLCHOLINE IN PC-EXCHANGED SYNAPTOSOMES

Synaptosomes (1.38 mg protein and 0.36 μmol PC/ml) were incubated with PC liposomes of the indicated fatty-acid composition (4.7–5.3 μmol PC/ml) and 30 units/ml of exchange protein for 3 h at 32°C in 0.32 M sucrose/10 mM glucose/20 mM Hepes (pH 7.4). A control sample was incubated with neither exchange protein nor liposomes. The synaptosomes, reisolated after the exchange by centrifugation, were extracted and analyzed for phospholipid and PC fatty-acid composition as described in Materials and Methods. The reported values are percentages of each fatty-acid species in the total PC fatty-acid pool. Liposome sticking was corrected for on the basis of the apparent increase in the synaptosomal PC pool size. Less than 16% of the sample phosphorus was referable to adhering PC liposomes in all cases. Values for the 'exchanged' fatty-acid species are underlined for clarity.

Fatty acid	Percentage of total fatty acid in the phosphatidylcholine for various types of liposome used in the exchange [a]							
	None	di(14:0) PC	di(16:0) PC	di(18:0) PC	di(18:1*cis*) PC	di(18:1*trans*) PC	di(16:0) PC + chol. [b]	di(18:1cis) PC + chol. [b]
14:0	–	49.4	–	–	–	–	–	–
16:0	51.0	26.3	58.0	48.9	24.8	34.5	60.5	23.9
16:1	trace	0.3	trace	trace	0.3	0.6	trace	trace
17:0	–	–	4.3	3.5	–	–	1.5	–
18:0	10.4	6.1	14.1	12.5	7.4	8.2	9.5	7.3
18:1 [c]	22.6	10.2	13.7	19.3	59.9	46.1	15.2	60.7
18:2	1.2	0.8	0.6	0.9	0.5	0.9	0.8	0.7
20:0	–	–	–	2.1	–	–	–	–
20:4	8.4	4.0	4.4	7.3	4.1	5.9	6.5	4.7
22:6	6.4	3.2	5.1	5.4	2.6	4.5	6.1	2.9
PC exchange (%) [d]	0	49.4	14.0	negligible	48.2	30.3	19.0	49.2

[a] The liquid-crystalline to gel-state transition temperatures of dimyristoyl (14:0), dipalmitoyl (16:0), distearoyl (18:0), dioleoyl (18:1*cis*) and dielaidoyl (18:1*trans*) PC are 23, 42, 58, below 0, and 9°C, respectively.

[b] Cholesterol (33 mol%) was included in these liposomes.

[c] Note that 18:1*cis* and 18:1*trans* methyl esters were not resolved by the gas chromatographic system employed (see Materials and Methods).

[d] The percentage of total PC fatty acid originating from the liposomes was calculated based on fatty-acid analysis as described in Materials and Methods, using the apparent increase in the synaptosomal PC pool size to correct for contaminating liposomes. This calculation takes into consideration replacement of endogenous synaptosomal PC fatty acids with liposomal PC fatty acids of like kind, assuming that synaptosomal PC's of all fatty-acid compositions are equally accessible to exchange. Thus in the case of exchange with di(14:0) PC, the increase in 14:0 fatty acid from 0 to 49.4% represents 49.4% PC exchange, whereas in the case of exchange with di(18:1)*cis* PC, the increase in 18:1 fatty acid from 22.6 to 59.9% represents $\{0.599-0.226(1-0.599)/(1-0.226)\}\cdot 100 = 48.2\%$ exchange.

change due to cholesterol in the liposomes could be compensated for by using longer (3 h) exchange incubation times (Table I). Initially, cholesterol (33 mol%) was included in the donor PC liposomes to assure that there was no net loss of synaptosomal membrane cholesterol by sterol partition into the PC liposomes. The spontaneous slow movement of sterols between liposomes and erythrocytes has been described by Bruckdorfer et al. [28]. However, the inclusion of cholesterol was found to be unnecessary, since the synaptosomal (and synaptosomal plasma membrane) cholesterol content was decreased less than 15% after several hours of incubation with PC liposomes which did not contain cholesterol.

In preparation for carrying out studies on the function of synaptosomes, exchange conditions had to be optimized to preserve synaptosomal integrity while allowing an adequate PC exchange rate. Glycerol, which was used for storage of the exchange protein at −20°C, had to be removed before incubation of the protein with the synaptosomes, since even 5% glycerol in the exchange medium (at 30°C) greatly reduced the synaptosomal membrane potential, measured using CC_5 fluorescence after reisolation of the synaptosomes. In addition, the synaptosomes remained most viable when provided with an exogenous energy source (10 mM glucose) while at near physiologic temperature (30–32°C), whether in 0.32 M sucrose

TABLE II

FATTY-ACID COMPOSITION OF PHOSPHATIDYLCHOLINE IN PLASMA MEMBRANES ISOLATED FROM SYNAPTOSOMES EXCHANGED WITH DISTEAROYL-PC, DIOLEOYL-PC AND DIELAIDOYL-PC

Synaptosomes (1.5 mg protein/ml) were incubated with distearoyl- (18:0), dioleoyl- (18:1*cis*) or dielaidoyl- (18:1*trans*) PC liposomes, containing 33 mol% cholesterol for 100 min at 32°C with or without exchange protein (24 units/ml) in Krebs-Hepes medium (pH 7.4) containing 0.2 M sucrose. The ratio of liposomal PC to synaptosomal PC was 6.6 in the case of the distearoyl-PC liposomes and 9.8 in the case of the dioleoyl- and dielaidoyl-PC liposomes. The synaptosomes were reisolated after the exchange, lysed and subfractionated according to the procedure of Jones and Matus [22], and the fatty-acid composition of PC in the synaptosomal plasma membrane fraction was determined as described in Materials and Methods. Liposome-sticking was accounted for on the basis of the apparent increase in the plasma membrane PC pool size. Less than 4% of the sample phosphorus was referable to adhering PC liposomes.

	Treatment					
Phospholipid exchange protein	–	+	–	+	+	+
Liposomes	–	–	Di(18:0) PC	Di(18:0) PC	Di(18:1*cis*) PC	Di(18:1*trans*) PC
Fatty acids	Percentage of total fatty acid in the phosphatidylcholine					
16:0	53.0	51.5	50.8	46.4	27.9	38.0
18:0	12.0	11.0	14.0	24.0	7.1	8.9
18:1 [a]	21.0	22.9	22.5	19.1	54.5	43.5
18:2	1.9	1.7	1.4	1.6	0.4	1.1
18:3/20:1	0.7	0.6	0.6	0.5	0.5	0.6
20:0	–	0.5	0.8	–	0.2	0.3
20:4	4.8	6.1	4.7	5.3	3.5	3.7
22:6	1.9	2.7	1.8	2.0	2.9	1.6
24:1	0.7	0.7	0.5	–	0.5	0.5
	Percentage of membrane PC exchanged					
Based on fatty-acid analysis [b]	0	0	2.2	15.0	42.0	28.5
Based on [^{14}C]PC exchange marker [c]	–	–	4.3	71.0	53.8	35.7

[a] Note that 18:1*cis* and 18:1*trans* methyl esters were not resolved by the gas chromatography system employed (see Materials and Methods).

[b] The percentage of membrane PC originating from the liposomes indicated by the observed alternations in fatty-acid composition (see the footnotes for Table I).

[c] The molar percentage of membrane PC which was ^{14}C-labeled, assuming that the ^{14}C-labeled 1-palmitoyl-2-oleoyl-PC exchange marker, which was present in all of the liposomes at 2 mol%, was exchanged into the synaptosomes at the same rate as the bulk pool of liposomal PC (i.e., the specific activity of the exchangeable PC in the liposome was identical to the specific activity of the liposomal PC as a whole). In this calculation, liposome-sticking was corrected for using [^{3}H]triolein..

or in physiologic saline (Krebs-Hepes). Since the exchange reaction was significantly faster in 0.32 M sucrose than in physiologic saline, the former was used usually unless the exchange was to proceed for more than 1 h, after which the synaptosomes in sucrose alone did not consistently retain their ability to generate or maintain a membrane potential.

We found that exchange with liposomal PC's in the liquid-crystalline state (dioleoyl-PC (42% exchange), dielaidoyl-PC (28.5% exchange) and dimyristoyl-PC (28% exchange)) did not alter the synaptosomal membrane potential or high-affinity uptake of choline and γ-aminobutyric acid (Table IV).

The kinetics of exchange of 1-palmitoyl-2-oleoyl-PC from a large excess of egg-PC liposomes into isolated synaptic plasma membranes was studied at 37°C. The exchange of this radiolabeled marker appeared to be representative of egg-PC

exchange into the synaptic membranes (c.f. Table II), since it is in the liquid-crystalline state at 37°C (T_m < 10°C) and is a major molecular species of egg PC [29], which undergoes a phase transition between −15 and −5°C [30]. In addition, it probably is a meaningful indicator of PC transbilayer movement within the fluid synaptic plasma membrane since it conforms well to bilayer structure (due to the relative lengths of its acyl chains). The observed kinetics of exchange, which behaved as the sum of two exponential components (Fig. 1), were applied to a simple model in which the synaptic plasma membrane PC exists in two pools, the inner and outer (exposed) portions of the bilayer. These pools are considered to be in equilibrium with each other and with the liposomes through the outer half of the bilayer only. The experiment is valid only when the membranes are in the form of sealed vesicles. Using this model,

TABLE III

FACTORS AFFECTING RATE OF PHOSPHATIDYLCHOLINE EXCHANGE INTO SYNAPTOSOMES

The effect of liposomal cholesterol and the presence of salt in the form of Krebs-Hepes medium (130 mM NaCl/5 mM KCL/1.3 mM $MgCl_2$/1.2 mM sodium phosphate/10 mM glucose/0.5 mM EGTA/10 mM Hepes, pH 7.4) on phosphatidylcholine exchange into synaptosomes was examined. Synaptosomes (1.75 mg protein/ml) were incubated with liposomes composed of egg PC or egg PC and cholesterol (2:1 or 1:1, molar ratio) in the presence of 40 units/ml exchange protein at 30°C for 40 min in either 0.32 M sucrose, 20 mM Hepes (pH 7.4) or Krebs-Hepes (pH 7.4) containing 0.2 M sucrose as indicated. The synaptosomes were reisolated for analysis after the exchange as described in Materials and Methods.

Liposome type [a]	Exchange medium	PC ratio (liposome/synaptosome) [b]	PC exchange (%) [c]
Egg PC	sucrose-Hepes	9.0	58
Cholesterol/egg PC (1:2)	sucrose-Hepes	8.9	31
Cholesterol/egg PC (1:1)	sucrose-Hepes	9.6	13
Cholesterol/egg PC (1:2)	sucrose-Hepes	4.6	21
Cholesterol/egg PC (1:2)	Krebs-Hepes-sucrose	4.6	8

[a] The liposomes contained 2 mol% 1-palmitoyl-2-oleoyl-[*choline-methyl*-^{14}C]PC and a trace of [^{3}H]triolein.

[b] Molar ratio of the liposomal and synaptosomal PC pools in the exchange mixture.

[c] The percentage of synaptosomal PC which was ^{14}C-labeled after the exchange, based on the specific activity of ^{14}C in the original liposomes and corrected for sticking (see Materials and Methods). Less than 8% of the sample phosphorus was referable to adhering liposomes in all cases. Although it is the comparative exchange of the ^{14}C-labeled 1-palmitoyl-2-oleoyl-PC marker which is most relevant here, it should also be noted that this exchange probably approximates bulk egg PC exchange from the liposomes since the marker PC, like egg PC, is above its phase transition temperature at 30°C. (See the comparison shown at the bottom of Table II between exchange of this marker and other liquid-crystalline PC's (dioleoyl and dielaidoyl).)

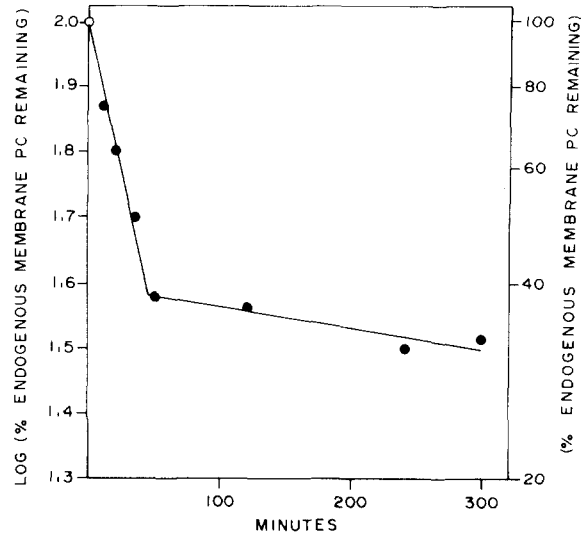

Fig. 1. Kinetics of phosphatidylcholine exchange between synaptic plasma membrane vesicles and an excess of egg PC liposomes. Synaptic plasma membranes (0.45 mg protein and 194 nmol PC/ml) were incubated at 37°C with egg PC liposomes (2194 nmol PC/ml) containing 5 mol% of ^{14}C-labeled 1-palmitoyl-2-oleoyl-PC and a trace amount of [^{3}H]triolein in the presence of 14 units/ml of exchange protein in 0.32 M sucrose, 70 mM Hepes (pH 7.4). The membranes were reisolated after the exchange by centrifugation through 0.5 M sucrose at the indicated time-points, and the fraction of membrane PC which was ^{14}C-labeled (and therefore originating from the liposomes) was determined. Liposome sticking, which increased the phosphorus content of the samples by 10–17%, was corrected for using the nonexchangeable marker, [^{3}H]triolein (see Materials and Methods). The logarithm of the percentage of endogenous PC remaining in the membranes (1 minus the fraction ^{14}C-labeled) was plotted versus the time, and the observed slopes and y-intercept were used to estimate the half-time for equilibration of PC between monolayers in the synaptic plasma membrane (10.2 h) and the fraction of the total membrane PC in the exposed face (0.63).

TABLE IV

THE EFFECT OF ALTERATIONS IN PLASMA MEMBRANE PC FATTY-ACID COMPOSITION ON SYNAPTOSOMAL FUNCTIONS

Synaptosomes (1.5 mg protein and 390 nmol PC/ml) were incubated with PC liposomes of the specified fatty acid composition (3.8 μmol PC/ml) containing 33 mol% cholesterol for 100 min at 32°C with or without exchange protein in Krebs-Hepes (pH 7.4) medium containing 0.2 M sucrose. The synaptosomes were reisolated after the exchange and assayed for membrane potential and choline and γ-aminobutyric acid (GABA) uptake. The remaining synaptosomes were lysed and subfractionated by the method of Jones and Matus [22]. The resultant synaptosomal plasma membranes were extracted and analyzed for phospholipid and PC fatty-acid composition. Liposome-sticking was corrected for on the basis of the apparent increase in the plasma membrane PC pool size. Less than 15% of the sample phosphorus was referable to liposome sticking in the case of dimyristoyl- (14:0) PC liposomes, and less than 4% in the case of the dioleoyl- (18:1*cis*) and dielaidoyl- (18:1*trans*) PC liposomes. n.m., not measured.

Liposomal PC	Exchange protein (units/ml)	Percentage PC exchange [a]	Fatty-acid alteration [b] (in PC)	Membrane potential ($\Delta F_{65\ mMK}/\Delta F_{5\ mMK}$) [c]	Choline uptake (pmol /min per mg) [d]	GABA uptake (pmol/min per mg) [d]
None	0	0	no change	0.197	8.2	39.2
	24	0	no change	0.209	8.2	40.0
Dimyristoyl-PC	0	0	no change	0.240	n.m.	n.m.
	30	28.2	0 → 28.2% (14:0)	0.245	n.m.	n.m.
Dioleoyl-PC	0	0	no change	0.264	10.5	40.9
	24	42.0	21 → 54.5% (18:1cis)	0.229	8.2	40.5
Dielaidoyl-PC	0	0	no change	n.m.	9.2	37.6
	24	28.5	0 → 28.5% (18:1trans)	n.m.	8.4	47.0

[a] The percentage of the total synaptosomal plasma membrane PC which originated from the liposomes, calculated on the basis of the observed alterations in fatty-acid composition (cf. footnotes at Table I).

[b] The alteration in the percentage of the indicated fatty-acid species in the total synaptosomal plasma membrane PC fatty-acid pool.

[c] Fluorescence intensity of the permeant cationic dye CC_5 was measured as described in Materials and Methods. $\Delta F_{5\ mMK}$ is the fluorescence change upon addition of the synaptosomes to the dye (2 μM) in Krebs-Hepes medium (pH 7.4) containing 0.2 M sucrose and 5 mM KCl. $\Delta F_{65\ mMK}$ is the change in fluorescence upon depolarization of the synaptosomes by raising the external K^+ concentration from 5 to 65 mM. $\Delta F_{65\ mMK}/\Delta F_{5\ mMK}$ is a qualitative index of the synaptosomal membrane potential existent in 5 mM K^+ medium (see Materials and Methods). An increasingly positive value for $\Delta F_{65\ mMK}/\Delta F_{5\ mMK}$ correlates with an increasingly negative membrane potential.

[d] Synaptosomal uptake of [^{3}H]choline (1 μM) and [^{14}C]GABA (1 μM) was measured as described in Materials and Methods.

the rate constants of the fast and slow exchange components and the relative size of the inner and outer pools of PC in the synaptic membrane vesicles were derived as described by Bloj and Zilversmit [25]. In this analysis, the relatively slow rate of PC translocation between outer and inner layers of the bilayer becomes limiting as exchange of label into the outer layer, limited in rate primarily by the amount of exchange protein present, nears completion. Within the membrane, boundary and bulk bilayer phospholipids are in rapid equilibrium, exchanging many times per s [31]. Therefore, immobilization of phospholipid by membrane proteins would not be expected to be rate-limiting in these phospholipid exchange experiments. Based on the biexponential plot of exchange in Fig. 1, the percentage of PC on the exposed face of the synaptic plasma membranes was estimated to be about 63%, with a half-time for equilibration of PC between outer and inner halves of the bilayer of about 10 h.

Discussion

The phosphatidylcholine exchange protein has been used to modify the fatty-acid composition of PC in synaptosomes and synaptic plasma membrane vesicles. Up to 50% of total synaptosomal PC can readily be exchanged with synthetic PC's in the liquid-crystalline state. Since approx. 40% of the synaptosomal phospholipid is PC, this corresponds to a 20% replacement of the original membrane phospholipid. The biphasic kinetics of PC exchange into synaptic plasma membrane vesicles (Fig. 1) indicated that the half-time of PC transbilayer equilibrium in these membranes was about

10 h. Hence, it appears that relatively little transbilayer movement of PC occurred within the plasma membrane of the synaptosome during the time frame of our experiments (0.5–3 h). In view of the slow transbilayer movement of PC, the observed 50% exchange of total synaptosomal PC probably represented nearly complete exchange of PC in the outer face of the synaptosomal plasma membrane. This extensive exchange could be accomplished without apparent loss of synaptosomal function, including membrane potential and high-affinity uptake of choline and γ-aminobutyric acid. Use of exchange proteins is the method of choice in the correlation of membrane-lipid composition with function, since the lipid changes can be accomplished using mild conditions and can be reversed to show restoration of altered function when it occurs [4].

The exchange protein strongly prefers that the PC to be exchanged into the synaptosomal membranes be in the liquid-crystalline state. PC's in the gel state could not be exchanged extensively into these membranes (Table I and II). Relevant to this observation, Kasper and Helmkamp [32] have reported that the PC exchange protein from bovine liver interacts preferentially with dimyristoyl-PC liposomes which are above rather than below the liquid crystal-to-gel phase transition. The synaptosomal plasma membrane, to which the synaptosomal membrane potential and choline and γ-aminobutyric-acid uptake are referable, is a fluid membrane containing a large percentage of polyunsaturated fatty acids. Since replacement of 20% of its phospholipid with exogenous PC's of fluid nature might not be expected to affect membrane properties significantly, it would have been informative to determine the effect of exchanged-in gel-state PC's on membrane function. Although this was not possible in our study with dipalmitoyl ($T_m = 42°C$) and distearoyl ($T_m = 58°C$) PC, it should be possible in the future with select PC's with transition temperatures near the physiologic range. Dimyristoyl-PC, with a transition at 23°C, is undesirable for these studies, since it does not form stable bilayers [33]. Suitable mixed-acid PC species which do form stable bilayers such as 1-palmitoyl-2-myristoyl-PC ($T_m = 27°C$) and 1-stearoyl-2-myristoyl-PC ($T_m = 33°C$) are now available by synthesis according to Eibl [15]. Their exchange could be accomplished above the transition temperature, while effects of the exchange on membrane function could subsequently be observed on either side of that temperature. There are practical limits to the extent of exchange which can be achieved for any phospholipid into synaptosomal membranes dictated by the slow kinetics of transbilayer equilibrium and the instability of the synaptosomes during prolonged incubations.

Our studies demonstrate that precaution must be taken in using a single radioactively labeled molecular species of PC as a tracer to follow the exchange of other types of PC molecules. Although this method of exchange monitoring has the significant advantage of convenience, it is of limited reliability when the marker is not identical with the bulk liposomal PC. The exchange protein is not random in its selection of PC molecules from mixed-PC liposomes. As seen in Table II, this problem was most obvious in the case of the fluid ^{14}C-labeled 1-palmitoyl-2-oleoyl-PC tracer in gel-state PC liposomes. However, exchange of this tracer was relatively representative of bulk PC exchange in liposomes composed of other liquid-crystalline PC's. The safest approach is to quantitate exchange by membrane fatty-acid analysis.

We were interested in determining the extent of exchange of intrasynaptosomal mitochondrial membranes, relative to the plasma membrane, during PC exchange of intact, functional synaptosomes. Unfortunately, this determination could not be done in a meaningful way by subfractionation of the synaptosomal fraction after exchange with liposomal PC, since a significant percentage of unsealed nerve endings, observable by electron microscopy, was present in the original synaptosomal preparation.

The biphasic kinetics of the exchange of ^{14}C-labeled 1-palmitoyl-2-oleoyl-PC from egg-PC liposomes to synaptic plasma membranes (Fig. 1) indicated that approx. 63% of the synaptic plasma membrane PC was exposed to the exchange protein without transbilayer movement. If it is assumed that essentially all of the membrane fragments were resealed into vesicles and were homogeneous in origin, a 63 : 37 transbilayer asymmetry for PC can be inferred as the minimum for that existing in the native orientation of the membrane.

This assumption is undoubtedly not entirely valid, although the membranes were largely in the form of sealed vesicles, based on their appearance in thin-section and freeze-fracture electron micrographs, exclusion of [^{14}C]dextran from a relatively large internal space, and ability to transport γ-aminobutyric acid [4]. The estimated half-time of PC transbilayer equilibrium in the synaptic plasma membranes (10 h) was similar to that measured in intact rat erythrocytes (labeled in vitro with ^{32}P) by Crain and Zilversmit [34] using a nonspecific lipid transfer protein from bovine liver.

Application of lipid transfer proteins to modification of the lipid composition of membranes is a relatively new development in the correlation of membrane lipid composition with function [4–8, 35]. Dyatlovitskaya et al. [6–8] have reported the use of a rat liver postmicrosomal supernatant to alter the phospholipid composition of mitochondrial and microsomal membranes. Crain and Zilversmit have used nonspecific and phosphatidylcholine-specific lipid transfer proteins to study the lipid dependence of glucose-6-phosphate phosphohydrolase [5]. They reported exchange of up to 43% of the total microsomal phosphatidylcholine with dipalmitoylphosphatidylcholine. This exchange was presumably evenly distributed across the microsomal bilayer due to the rapid transbilayer movement of phospholipid in this membrane [36]. Recently, we reported use of the nonspecific transfer protein from bovine liver to alter the cholesterol-to-phospholipid ratio of synaptosomes and synaptic plasma membranes, and observed a specific and reversible loss of γ-aminobutyric-acid uptake upon lowering of membrane cholesterol-to-phospholipid ratio [4]. In the present study, we describe the use of an exchange protein to specifically catalyze maximal exchange of synaptosomal membrane phosphatidylcholine with synthetic phosphatidylcholines of defined fatty-acid composition. A near complete exchange of phosphatidylcholine in the outer face of the synaptosomal plasma membrane was accomplished without loss of synaptosomal membrane potential or high-affinity uptake of choline and γ-aminobutyric acid.

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