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PROTEIN-STIMULATED ENRICHMENT OF HUMAN PLATELET MEMBRANES IN LINOLEYL-PHOSPHATIDYLCHOLINES

EFFECT UPON ADENYLATE CYCLASE AND FLUIDITY

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In order to study the effect of linoleyl enrichment of platelet membranes upon adenylate cyclase activity and membrane fluidity, manipulations of platelet phospholipids are carried out with phosphatidylcholine-loaded high-density lipoproteins (HDL) or phospholipid-exchange protein and phospholipid-cholesterol mixed vesicles. Incubation with HDL does not appear to be valuable for this purpose. On the other hand, phospholipid-exchange protein and mixed vesicles can be used successfully. Phospholipid-exchange protein stimulated 3-fold the spontaneous exchange of 2-linoleylphosphatidylcholine between the vesicles and the platelets. Linoleyl enrichment of platelets by dilinoleylphosphatidylcholine is about 25% and by 2-linoleylphosphatidylcholine is about 45–50%. The unsaturation index remains constant when the enrichment is performed using dilinoleylphosphatidylcholine but it increases with 2-linoleylphosphatidylcholine. Basal and prostaglandin E_1-stimulated adenylate cyclase activities are not modified by dilinoleylphosphatidylcholine, while they increase significantly in the case of 2-linoleylphosphatidylcholine. There is no significant variation in diphenyl hexatriene fluorescence polarization parameters, either with dilinoleylphosphatidylcholine or with 2-linoleylphosphatidylcholine.

Introduction

Joist et al. [1] in rabbit and ourselves [2] in man have demonstrated that platelets are able to exchange phospholipids with plasma lipoproteins. The assembly and secretion of the plasma lipoproteins occurs only in the liver and the intestine [3]. Several authors have established that saturated and polyunsaturated fatty acid-rich diets have opposite effects upon platelet functions in man [4,5]. Studying the effect of long-term feeding of dietary fats upon platelet functions, Renaud and colleagues [6] showed recently that platelet aggregation and clotting activity of human platelet-rich plasma were significantly correlated only with the saturated fat intake.

Platelet activation is triggered in the end by mobilization of calcium from platelet granules [7] and calcium storage in platelet granules is modulated by cyclic AMP [8]. Several authors have attempted to modify adenylate cyclase activity and/or membrane fluidity by modulating the fatty acyl composition of LM cells [9,10], hepatocytes [11–13], fibroblasts [14], lymphocytes [15] and mutant hamster ovary cells [16]. At the present time it has been shown that cholesterol-enriched platelets have an increased sensitivity to epinephrine [17], with an increased membrane micro-

Abbreviations: DLPC, dilinoleylphosphatidylcholine; DPH, 1,6-diphenyl-1,3,5-hexatriene.

viscosity [18] but controversial changes in the adenylate cyclase activity [19,20].

According to Renaud's work, in which the membrane content in cholesterol was unchanged [6], one could hypothesize that dietary fatty acids interact to some degree with platelet functions through a plasma membrane modification. The major molecular species of plasma phospholipids are 1-palmityl (or stearyl)-2-oleyl-*sn*-glycero-3-phosphocholine and 1-palmityl (or stearyl)-2-linoleyl-*sn*-glycero-3-phosphocholine. As phospholipids with oleyl in the 2-position constitute the predominant molecular species of the exchangeable pool of platelet plasma membrane [21], the manipulation of platelets with oleyl molecular species would have resulted in exchanging oleyl versus oleyl.

In this paper we have investigated the possibility of enriching platelet membranes with 1-palmityl-2-linoleyl-*sn*-glycero-3-phosphocholine and with 1-linoleyl-2-linoleyl-*sn*-glycero-3-phosphocholine, a phospholipid already known to possess an inhibitory effect on platelet aggregation [22]. We have used both high-density lipoproteins (HDL) and phospholipid-exchange protein to modulate phospholipid composition of platelet membranes. Platelet contaminatoin by vesicles occurred to too large an extent during incubations of washed platelets with HDL, and this did not allow us to control efficiently modifications of the fatty acyl composition of platelet membranes. Phospholipid-exchange protein are proteins known to stimulate the specific transfer of phospholipid molecule between membranes or vesicles [23]. They were only recently used for phospholipid-manipulating studies with protoplasts [24] and with human erythrocyte plasma membrane [25,26]. Incubations of washed platelets with phospholipid vesicles in the presence of phospholipid-exchange protein gave successful results which have allowed us to study the effect of linoleyl-enrichment of platelet membranes upon adenylate cyclase activity and membrane fluidity.

This work was presented in part at the 23rd International Conference on the Biochemistry of Lipids, Copenhagen, August 1981.

Materials and Methods

Preparative procedures

Synthesis of 1-acyl-2-linoleyl-sn-glycero-3-[^{32}P]-phosphocholine. Labeled phosphatidylcholine was prepared by injecting three rats intraperitoneally with [^{32}P]orthophosphate (5 mCi) 18 h before killing. Livers were sliced extracted with chloroform/methanol (1 : 2, v/v) and the extract was washed using the procedure of Folch [27]. Phosphatidylcholine was then isolated by silicic acid column chromatography. Phosphatidylcholine purity was controlled by thin-layer chromatography.

The [^{32}P]phosphatidylcholine was hydrolyzed 16 h at room temperature with snake venom in diethyl ether [28], which precipitates lysophosphatidylcholine. The precipitate was washed six times with diethyl ether, dried under nitrogen and dissolved in chloroform. Linoleic acid anhydride (714 μmol) was prepared according to Salinger [29], filtered and controlled by TLC using the petroleum ether/diethyl ether (8 : 1, v/v) system. [^{32}P]Lysophosphatidylcholine (60 μmol) dissolved in 5 ml anhydrous chloroform/pyridine (4 : 1, v/v) was added under nitrogen to dried linoleic acid anhydride. Reacylation was initiated by adding dimethylaminopyridine as a catalyst in a large excess with regard to lysophosphatidylcholine [30]. Successive thin-layer chromatographies were performed to determine the time required for complete reacylation, which was achieved after 24 h incubation. Starting with 110 μmol of liver-extract [^{32}P]phosphatidylcholine we have obtained 20 μmol of purified 1-acyl-2-linoleyl-*sn*-glycero-3-phosphocholine. Using the same technique we have obtained 1-acyl-2-[1-^{14}C]linoleyl-*sn*-glycero-3-phosphocholine for phospholipase assay.

Vesicles. In HDL experiments we prepared vesicles from 60 mol pure dilinoleylphosphatidylcholine (DLPC) (L-α-dilinoleyl-*sn*-glycero-3-phosphocholine, Sigma, St. Louis, MO). In phospholipid-exchange protein experiments we prepared mixed vesicles with DLPC (60 μmol) or with 1-acyl-2-linoleyl-*sn*-glycero-3-[^{32}P]phosphocholine (20 μmol) and with cholesterol and cholesteryl[1-^{14}C]oleate (69 μCi/μmol) or [^{14}C]triolein (60 μCi/μmol) as radiotracers (New England Nuclear). The lipid mixture dissolved in chloroform was dried and then resuspended in diethyl

ether (100 μl) and 4.5 ml Tris buffer (20 mM Tris/0.9% NaCl (pH 7.5)) were added. Butylated hydroxytoluene (0.1%, w/v) was added and the suspension was sonicated (MSE 150 W ultrasonic desintegrator) under nitrogen for 30 min at 75% power. During sonication the lipid suspension was at 2°C, a temperature well above the thermotropic transition maintained thermostatically for these highly unsaturated phosphatidylcholines.

Non-dispersed phospholipid and titanium particles were removed by centrifugation for 30 min at 48000 × *g*. The lipid composition of the resulting vesicles was checked by lipid phosphorus cholesterol content and specific radioactivity. Vesicular solution was never stocked more than 1 or 2 days under nitrogen at the sonication temperature.

Loading of HDL with specific vesicles. High-density lipoproteins (HDL) were isolated from serum by precipitation as described by Burnstein et al. [31]. Their phospholipid/protein ratio (0.5, w/w) was determined and their linoleic acid content (31 mol%) was measured by GLC.

HDL were incubated with dilinoleylphosphatidylcholine vesicles (lipid : phosphorus ratio 1 : 3) for 1 h at 37°C under mild agitation. Loading with HDL is followed by a gradual decrease in density. Thus, to remove free vesicles, HDL were centrifuged at density of 1.05 (140000 × *g*, 24 h). A second centrifugation was carried out at density 1.21 to discriminate the loaded HDL from their native HDL. Loaded HDL were then dialyzed against Tris-NaCl buffer (20 mM Tris/0.9% NaCl (pH 7.4)) and kept at −20°C for a few days.

The loading of HDL with DLPC was determined by measuring their phospholipid : protein ratio (0.8, w/w) and their linoleic acid content (55 ± 6 mol%).

Phospholipid-exchange protein. According to Wirtz and Zilversmit [32] phospholipid-exchange protein was prepared from rat liver cytosol. The pH 5.1 supernatant fraction contained about 0.02 μg phospholipid phosphorus per mg protein. After adjustment to pH 7.4, crude phospholipid-exchange protein preparation was stored at −20°C.

Phospholipid transfer activity of the phospholipid-exchange protein preparation was determined using liposomes as the donor membrane and mitochondria as the acceptor membrane [33]. Rat liver mitochondria (2.5 mg protein, 0.55 μmol phospholipids) and mixed liposomes (87000 cpm ^{32}P, 0.25 μmol phosphatidylcholine; 93000 cpm ^{14}C, 0.4 nmol cholesterol oleate) were incubated with or without the pH 5.1 supernatant (5 mg protein) in 3 ml Tris buffer (pH 7.4) for 1 h at 37°C. ^{14}C recovered with mitochondria after incubation without phospholipid-exchange protein permitted estimation of the contamination of mitochondria by liposomes. Under these conditions the rate of exchange was 8 nmol/h. 5 mg phospholipid-exchange protein gave an exchange of $5 \times 8 = 40$ nmol phosphatidylcholine. As the amount of vesicle phospholipids was 0.25 μmol, the percentage of exchange has been calculated as:

$$\frac{\text{exchanged phospholipids}}{\text{total phospholipids}} = \frac{40}{250}$$

= 16% of the vesicle phosphatidylcholine

Zilversmit, with roughly similar conditions, found 25% [33].

Because ^{14}C found with mitochondria increased upon phospholipid-exchange protein addition, we conducted further purification by ammonium sulfate precipitation (pH 7.4) after concentration in a PM 10 cell under nitrogen. The precipitate redissolved, dialyzed against phosphate buffer (0.15 M phosphate/1 mM β-mercaptoethanol (pH 7.4)) was further concentrated and applied to Sephadex G-75 column chromatography as described by Wirtz and Zilversmit [34]. Phospholipid-exchange protein preparation no longer exhibited cholesterol-ester exchange activity after the chromatographic step (Table I). In most experiments we have used the phospholipid-exchange protein preparation obtained after ammonium sulfate precipitation.

Platelet preparation. The Centre National de Transfusion Sanguine provided us with fresh blood collected according to Aster and Jandl [35] from young male donors (20–30 years old, A^+ group) who had not taken any drug within the week.

Blood was centrifuged 20 min at 190 × *g*, platelet-rich plasma was removed and further centrifuged for 15 min at 2500 × *g*. The platelet-poor plasma was discarded and the platelet pellet resuspended in Tyrode solution containing albumin and apyrase as described by Mustard et al.

TABLE I

ACTIVITY OF RAT LIVER PHOSPHOLIPID-EXCHANGE PROTEIN AT DIFFERENT STEPS OF ITS PREPARATION AND PURIFICATION

Phospholipid exchange activity of phospholipid-exchange protein (PLEP) is determined using rat liver mitochondria (2.5 mg protein; 550 nmol phospholipids) as acceptor membrane. They are incubated in tyrode/apyrase buffer (pH 7.4) for 1 h at 37°C with 2-linoleyl[^{32}P]phosphatidylcholine/cholesterol/cholesterol[1-^{14}C]oleate (250 nmol phospholipids; 2 : 1 : 0.006) (87000 cpm ^{32}P; 93000 cpm ^{14}C). Amounts of phospholipid-exchange protein derived from 5 mg proteins of the pH 5.1 supernatant are added to stimulate the exchange. Contamination of mitochondria by liposomes is estimated by ^{14}C cpm recovered with mitochondria. The amount of contaminating phospholipids is calculated by multiplying vesicle specific ^{32}P radioactivity by the percentage of ^{14}C recovered with mitochondria. The amount of exchanged phosphatidylcholine is calculated as:
(% ^{32}P cpm in mitochondria − % ^{14}C cpm in mitochondria) × vesicle phospholipid.

Preparation steps	Proteins (mg)	% ^{14}C cpm (mitochondria)	% ^{32}P cpm (mitochondria)	Phospholipid exchange (nmol/mg)
pH 5.1 supernatant	2400	23.8	39.8	8.0
PM 10 cell concentrate	1900	18.2	37.6	9.7
$(NH_4)_2SO_4$ precipitate	1000	17.0	46.0	14.5
Sephadex G-75	42	15.3	54.1	19.4
Mitochondria + liposomes without PLEP	–	14.2	18.2	–

[36]. All operatoins were carried out at 37°C under CO_2. Platelets were then counted in a Malassez cell. Measurements of platelet functional integrity were performed by aggregometry and serotonin release.

Incubations and analytical procedures

Incubation with loaded HDL. Platelet suspensions (8 ml) were incubated with DLPC-loaded HDL (5 mg HDL protein, i.e., 5–8 μmol HDL phospholipids per 10^9 platelets (0.58 ± 0.2 μmol phospholipid)) for 1 h at 37°C under CO_2 without agitation. Platelet suspensions were incubated to control the platelet activities under the same conditions without DHL.

After the incubation period, platelets were washed three times in Tyrode buffer. Aliquots were used immediately to test platelet activities; others were frozen in liquid nitrogen and stored at −70°C for adenylate cyclase assay and fluorescence polarization measurements. Finally, aliquots were washed once more in Tris/NaCl buffer to discard albumin and proteins were measured and lipid extracted by methanol/chloroform (2 : 1, v/v).

Incubations with mixed vesicles in presence of phospholipid-exchange protein. Platelet suspensions (8 ml) were incubated with mixed vesicles (1 μmol vesicle phospholipid per 10^9 platelets; 0.58 ± 0.2 μmol phospholipid) in the presence or in the absence of phospholipid-exchange protein (5 mg per 10^9 platelets). After 1 h incubation at 37°C under CO_2 without agitation, platelets were prepared as mentioned above.

Lipid and protein analysis. Lipid extraction was carried out with methanol/chloroform (2 : 1, v/v). TLC was conducted on silicagel G in chloroform/methanol/water (65 : 25 : 4 v/v). Spots were revealed by iodine vapor and/or radioautography. Glycerolipids were hydrolyzed with 0.5 M methanolic KOH for 5 min, followed by a BF_3 methanolic transmethylation for 5 min at 60°C under nitrogen [37]. Fatty acids were measured by GLC using a 3 m column filled with 4% diethyleneglycol succinate on Gas-chrom Q (100–120 mesh) operated at 170°C and detected by flame ionization.

Lipid phosphorus was measured according to Rouser et al. [38], cholesterol according to Kim and Goldberg [39] and proteins were assayed

according to Lowry et al. [40]. Radioactivity was measured using liquid scintillation. Quenching was evaluated by means of the interval channel ratio method [41].

Platelet aggregometry. Platelet aggregation was studied using an Elvi Aggregometer. The test was carried out firstly with 0.25 ml platelet-rich plasma for controlling fresh platelet aggregability. After the incubation time, tests were carried out with resuspended platelets in a volume of 0.25 ml Tyrode buffer. Aggregating agents (thrombin, ADP, arachidonate) were added in 5 μl as described elsewhere [42].

Platelet serotonin release. Platelets were prelabeled firstly with [^{14}C]serotonin (5-hydroxytryptamine 1-[^{14}C]formiate 49 Ci/mol, Commissariat à l'Energie Atomique, Gif sur Yvette, France) as described by Mustard et al. [43]. Release of [^{14}C]serotonin into the suspending fluid was measured 5 min after the addition of thrombin (1 U per 1 ml platelet suspension) according to Greenberg et al. [44].

Adenylate cyclase assay. Platelet lysates were prepared by successive freezing-thawing steps and kept on ice until assay. Assays were performed at 37°C in 0.1 ml medium containing 50 mM Tris-HCl (pH 7.4), $1.5 \cdot 10^6$ dpm [α-^{32}P]ATP (40 Ci/mmol, provided by Amersham France, Versailles, France), 5 mM $MgCl_2$, 1 mM isobutylmethylxanthine (IBMX); 1 mM cyclic AMP, 1 mM EGTA and ATP-regenerating system consisting of 5 mM creatine phosphate and 1 mg/ml creatine kinase.

Incubations were initiated by the addition of platelet lysate (25–100 μg protein) carried out in triplicate for 10 min and terminated by addition of 0.2 ml 1 M HCl. Approx. 10000 cpm cyclic [^{3}H]AMP (Amersham International, U.K.) were added to monitor [^{32}P]-cAMP recovery. After addition of water to 1 ml final volume, cyclic AMP was isolated by several sequential passages of the sample over Dowex and Alumina columns according to the method of Salomon et al. [45]. The final 3 ml eluate were directly collected into a scintillation vial and counted adding 10 ml Lumagel. The data were obtained under conditions where the cyclic AMP formation is linear with respect to time and protein concentration. Prostaglandin E_1 was a gift from Dr. Pike (Upjohn Co, Kalamazoo, MI).

Fluorescent-polarization measurements. The fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) (Sigma, St. Louis, MO) was used to label platelet membranes (the particulate fraction from freezing-thawing lysate) as described by Shatill and Cooper [18]. Fluorescence intensities were detected with a Jobin Yvon JYE spectrofluorometer through a polarizer oriented parallel and perpendicular to the direction of the polarized excitation beam. The steady-state fluorescence anisotropy is r:

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}}$$

and the apparent microviscosity is estimated according to the Perrin equation (Shinitzky and Barenholz [46])

$$\frac{r_0}{r} = 1 + C(r)\frac{T \cdot \tau}{\bar{\eta}}$$

where r_0 is the upper limit of $I_{\parallel}/I_{\perp}$ ($r_0 = 0.362$). τ is the lifetime of the excited state, $C(r)$ is a structural parameter of the probe which varies slightly with r and was calibrated in white oil for DPH according to Shinitzky and Inbar [47]. T is the absolute temperature.

The fusion activation energy for microviscosity (ΔE) was calculated from the slope of the plot of $\log \bar{\eta}$ vs. $1/T$. The lifetime of the excited state (τ) was measured with the aid of Drs. Vincent and Galley (Etats Moléculaires liés, Faculté des Saints-Pères, Paris) with the single photon device (Ortec) assuming a biexponential decay for DPH. The average fluorescence lifetime was used for calculation of the apparent microviscosity.

Results

Linoleyl enrichment of platelets

The enrichment of platelets in linoleic acid from dilinoleylphosphatidylcholine was firstly studied with high-density lipoproteins (HDL) as donor. Immunoelectrophoretic properties of loaded HDL differed from those of native HDL. This may be explained by a partial redistribution of HDL apoproteins between HDL and vesicles as shown by many authors [48,49]. Nevertheless, when HDL are incubated with ^{32}P-labeled phosphatidylcho-

line the radioactivity is recovered with apoprotein lines on immunoelectrophoretograms as revealed by radioautography [2]. These data assess that if loaded HDL are different from native HDL, they are stable lipoprotein structures efficient for exchange and are not trivial aggregates [48].

Table II displays the linoleyl enrichment of platelets from DLPC-HDL. After 1 h incubation the platelet level of linoleic acid rises from 10.1 ± 0.5 to 11.9 ± 0.6 mol%. But the starting level is already 30% higher than the platelet linoleyl level before the addition of loaded HDL (7.8 ± 1.2 mol%), which indicates an instantaneous non-specific interaction and/or contamination of the platelets. These results show that HDL cannot be used successfully in these studies with washed platelets, in contrast to previous studies with platelet-rich plasma [2].

This prompted us to choose the dilinoleylphosphatidylcholine vesicles as donor and phospholipid-exchange protein to stimulate the transfer. Cholesterol transfer between membranes and vesicles occurs according to the concentration ratio in the presence as well as in the absence of phospholipid-exchange protein [50]. Therefore, to rule out any modification of platelet cholesterol content we have incubated platelets with mixed vesicles of phosphatidylcholine-cholesterol in the same molar ratio (2 : 1) as platelet plasma membranes.

TABLE II

LINOLEYL ENRICHMENT OF PLATELETS FROM DILINOLEYLPHOSPHATIDYLCHOLINE-LOADED HDL

DLPC-loaded HDL (5–8 μmol of HDL phospholipid per 10^9 platelets) are added to platelets for 0 or 1 h. After incubation the platelets are washed twice to discard HDL, then the lipids are extracted for analysis. Results are expressed as mean percentage ± S.D. ($n = 15$).

Platelets	+	+	+
DLPC-loaded HDL	0	+	+
Incubation time	0	0	1 h
Linoleic acid (mol%)	7.8 ± 1.2	10.1 ± 0.5 [a] (+30%)	11.9 ± 0.6 [a] (+52%)

[a] $P < 0.001$.

TABLE III

OCCURRENCE OF A CHOLESTEROL-ESTER TRANSFER ACTIVITY IN THE PHOSPHOLIPID-EXCHANGE PROTEIN PREPARATION

Platelets ($1 \cdot 10^9$) are incubated for 1 h in presence or absence of phospholipid-exchange protein (PLEP) (equivalent to 5 mg pH 5.1 supernatant proteins) with mixed phospholipid-cholesterol vesicles (1 mol; 2 : 1) containing as tracer cholesterol [1-^{14}C]oleate (980000 cpm) or [^{14}C]triolein (999000 cpm). After incubation, platelet radioactivity is measured on washed platelets. n.s., not significant. Results are presented in dpm or (% of total radioactivity).

Incubation	Cholesterol [1-^{14}C]oleate	[^{14}C]Triolein
Without PLEP	4180 ± 360 (0.44 ± 0.04%)	4240 ± 365 (0.43 ± 0.04%)
With PLEP	4900 ± 150 (0.50 ± 0.02%)	4205 ± 310 (0.42 ± 0.03%)
	$P < 0.05$	n.s.

Despite the fact that PLEP preparations used for phospholipid exchange exhibited a cholesterol-ester transfer activity (Tables I and III), we can calculate absorption and/or endocytosis with this marker. Table III shows the same contamination without phospholipid-exchange protein when measured with cholesterol [1-^{14}C]oleate or [^{14}C]triolein. Further phospholipid-exchange protein preparation does not increase [^{14}C]triolein in platelets after a 1 h incubation at 37°C. Thus, the percentage of cholesterol [1-^{14}C]oleate found with platelets (or with mitochondria) when incubations are carried out without phospholipid-exchange protein allows us to calculate the contamination by vesicles, b, as:

$$b = \text{nmol phosphatidylcholine in vesicles} \times \frac{\text{dpm } ^{14}\text{C in platelets without phospholipid-exchange protein}}{\text{dpm } ^{14}\text{C in the whole incubation medium}}$$

The phospholipid-exchange protein preparation used for exchange has no phospholipase activity (Table IV). Fatty acid gas-liquid chromatograms

TABLE IV

ABSENCE OF PHOSPHOLIPASE ACTIVITY IN THE PHOSPHOLIPID-EXCHANGE PROTEIN PREPARATION USED IN THE EXCHANGE EXPERIMENTS

1-Acyl-2-[1-^{14}C]linoleyl-*sn*-glycero-3-phosphocholine vesicles (2 μmol, $1.6 \cdot 10^6$ cpm) are incubated with or without $(NH_4)_2SO_4$ precipitate obtained from pH 5.1 supernatant (10 mg protein) for 1 h in 5 ml of the buffer used for experiments with platelets. A control is carried out with *Crotalus* venom phospholipase A_2 (Sigma) added in the sucrose buffer of phospholipid-exchange protein (PLEP) preparation. Results are expressed as mean percentages ($n = 3$) of total radioactivity found with free fatty acids or phosphatidylcholine after thin-layer chromatography of incubation medium extracts.

Vesicles	+	+	+
PLEP	0	+	0
Phospholipase A_2	0	0	+
Buffer	+	+	+
Free fatty acids	0.68 ± 0.60	0.37 ± 0.40	64.53 ± 0.45
Phosphatidylcholine	99.30 ± 0.60	99.60 ± 0.70	35.12 ± 1.00

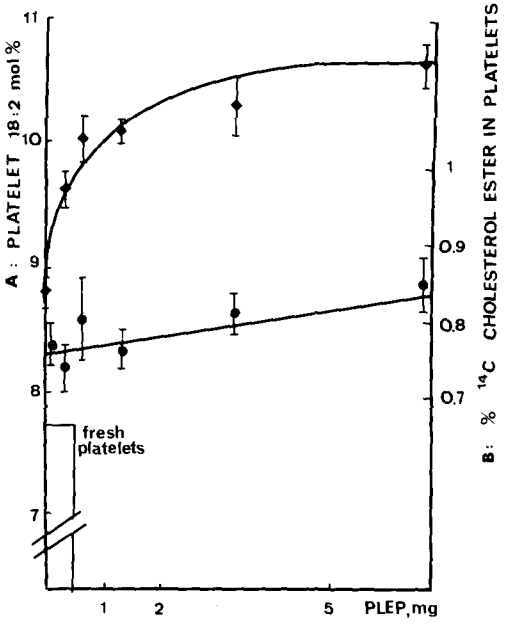

Fig. 1. Phospholipid-exchange protein-stimulated linoleyl enrichment of platelets from mixed vesicles of dilinoleylphosphatidylcholine/cholesterol (2 : 1). Platelets ($1 \cdot 10^9$) are incubated for 1 h at 37°C with mixed vesicles of dilinoleylphosphatidylcholine-cholesterol/cholesterol[1-^{14}C]oleate (1 μmol phosphatidylcholine; 2 : 1 : 0.003) in the presence of ammonium sulfate purified phospholipid-exchange protein preparation which originates from 0 to 6.6 mg protein of supernatant (pH 5.1). Cholesterol oleate allows us to estimate the contamination of platelets by vesicles. Results are expressed as mean percentages ± S.D. ($n = 3$). A: platelet content in 18 : 2 (mol%): ◆; B: Vesicle contamination as percentage of the total vesicles present in the incubation medium (●):

$$B = \frac{^{14}\text{C dpm in platelet after incubation}}{\text{total } ^{14}\text{C dpm in vesicles before incubation}} \times 100$$

of the main platelet phospholipids are displayed in Table V. Only phosphatidylcholine exhibits a modification of the fatty acid pattern when phospholipid-exchange protein is added in the incubation medium. When we used ^{32}P-labeled platelets, more than 90% of the radioactivity was found in phosphatidylcholine in the incubation medium. We could not find any trace of other phospholipids by measuring the lipid phosphorus after thin-layer chromatography.

In Fig. 1 can be seen the linoleyl enrichment of platelets from mixed vesicles with increasing amounts of phospholipid-exchange protein. When platelets are incubated with vesicles but without phospholipid-exchange protein, the linoleic acid level is higher than the initial level in fresh platelets: 8.1 ± 0.1 vs. 7.7 ± 0.2 mol%. With 5 mg phospholipid-exchange protein, the increase reaches 10.7 ± 0.2 mol%.

As dilinoleylphosphatidylcholine is a very minor molecular species of plasma phosphatidylcholines we have carried out the same experiments with ^{32}P-labeled 2-linoleylphosphatidylcholine. These experiments allow us to estimate platelet enrichment in linoleic acid close to the physiological conditions (Table VI). In the absence of phospholipid-exchange protein we evidence both spontaneous exchange of phosphatidylcholine (17 nmol) and contamination (11.0 nmol). With 5 mg phospholipid-exchange protein the exchange is stimulated 3-times.

The contamination is reduced, furthermore, by

TABLE V

FATTY ACID COMPOSITION OF PLATELET PHOSPHOLIPIDS

Platelets ($1 \cdot 10^9$) are incubated for 1 h at 37°C with mixed vesicles of 2-linoleyl[^{32}P]phosphatidylcholine/cholesterol/cholesterol [1-^{14}C]oleate (1 μmol, 2 : 1 : 0.003) in the presence of 5 mg phospholipid-exchange protein. After washing, platelet pellets are extracted and phospholipids separated by TLC. Fatty acid content of each phospholipid is measured by GLC after elution, hydrolysis and transmethylation of phospholipids spots. Results are expressed as mol% of total fatty acids ± S.D. ($n = 3$).

Platelets		+	+	+
Vesicles		0	+	+
Phospholipid-exchange protein		0	0	+
Phosphatidylcholine	16 : 0	26.4 ± 4.2	33.5 ± 3.3	32.8 ± 2.0
	16 : 1	0.9 ± 0.1	1.7 ± 0.1	2.3 ± 2.1
	18 : 0	24.5 ± 5.5	24.9 ± 2.3	21.7 ± 3.6
	18 : 1	29.7 ± 5.0	24.1 ± 0.9	19.5 ± 1.0
	18 : 2	8.6 ± 1.3	9.3 ± 0.5	19.1 ± 0.9
	20 : 4	8.9 ± 1.4	8.2 ± 0.9	4.0 ± 1.4
Phosphatidylethanolamine	16 : ald	4.6 ± 2.4	6.1 ± 3.2	2.6 ± 1.3
	16 : 0	8.2 ± 1.8	8.7 ± 2.1	7.4 ± 1.6
	16 : 1	2.4 ± 0.5	2.8 ± 0.9	0.9 ± 0.4
	18 : ald	10.0 ± 0.8	10.7 ± 1.2	11.7 ± 1.1
	18 : 0	23.6 ± 2.7	20.7 ± 3.2	24.2 ± 2.1
	18 : 1	12.8 ± 1.2	13.6 ± 2.1	9.5 ± 1.7
	18 : 2	4.0 ± 1.0	3.6 ± 0.8	4.0 ± 0.7
	20 : 4	34.4 ± 3.2	33.8 ± 3.6	39.7 ± 4.1
Phosphatidylserine + phosphatidylinositol	16 : 0	21.8 ± 1.1	21.9 ± 1.0	21.9 ± 0.7
	16 : 1	1.8 ± 0.5	1.4 ± 0.1	1.6 ± 0.9
	18 : 0	23.8 ± 3.1	22.3 ± 4.3	25.5 ± 1.5
	18 : 1	17.3 ± 1.6	18.2 ± 3.0	15.9 ± 2.5
	18 : 2	15.0 ± 2.2	16.3 ± 0.1	15.5 ± 3.0
	20 : 3	2.1 ± 0.8	2.2 ± 0.6	2.3 ± 1.2
	20 : 4	18.2 ± 4.4	17.8 ± 3.7	17.6 ± 5.2

carrying out incubations at 2°C, which inhibits endocytosis of vesicles [52]. Under these conditions the contamination is reduced by 50% but at the same time the stimulatory effect of phospholipid-exchange protein upon exchange is also inhibited.

Effect on platelet adenylate cyclase

Activities observed with control platelets are close to those published by Jakobs et al. [53] and Cooper et al. [54]: respectively 6.7 ± 0.3, 10.8 and 7.0 ± 0.8 pmol/min per mg protein.

Table VII shows that neither the basal activity nor the activity stimulated by $1 \cdot 10^{-6}$ M prostaglandin E_1 is modified in dilinoleylphosphatidylcholine-enriched platelets with regard to control. On the other hand the activity stimulated by $1 \cdot 10^{-2}$ M fluoride is significantly higher than control ($P < 0.1$). Although the linoleyl enrichment of platelets from DLPC is 25% of the original platelet linoleic acid, the unsaturation index of platelets (number of double bonds per 100 fatty acid) is not modified.

With 2-linoleylphosphatidylcholine vesicles under all three conditions, the adenylate cyclase activities are affected by the phospholipid exchange. The basal activity ($P < 0.005$) and activity stimulated by fluoride ($P < 0.05$) or by prostaglandin E_1 ($P < 0.05$) are significantly higher in the linoleyl-enriched platelets than in the control platelets. We also observe a significant increase in the unsaturation index ($P < 0.001$) after the exchange. For each experiment, the viability of platelets has been controlled by testing their ability to release

TABLE VI

PHOSPHOLIPID-EXCHANGE PROTEIN-STIMULATED LINOLEYL EXCHANGE WITH PLATELETS AND MIXED VESICLES OF 2-LINOLEYLPHOSPHATIDYLCHOLINE/CHOLESTEROL (2:1)

Platelets (10^9) are incubated for 1 h at 37°C with mixed vesicles of 2-linoleyl[^{32}P]phosphatidylcholine/cholesterol/cholesterol [1-^{14}C]oleate (1 μmol, 2:1:0.003) (51500 cpm ^{14}C; phosphatidylcholine-specific radioactivity 335 ^{32}P cpm/nmol) in the presence of 5 mg phospholipid-exchange protein (PLEP). After washing, platelet content in fatty acids is measured by GLC. Adsorbed or internalized phosphatidylcholine is b:

$$b = \frac{\text{total } ^{32}\text{P cpm}}{\text{phosphatidylcholine } ^{32}\text{P s.a.}} \cdot \frac{a}{\text{total } ^{24}\text{C cpm}}$$

where a is ^{14}C cpm associated with platelets in the absence of PLEP (5700 ± 1800). s.a., specific activity. The exchanged phosphatidylcholine is derived from platelet cpm as c:

$$c = \frac{\text{platelet } ^{32}\text{P cpm}}{\text{phosphatidylcholine } ^{32}\text{P s.a.}} - b$$

Platelet membrane 18:2 content is estimated as d:
d = platelet phospholipid content × 2 × platelet 18:2 content.

Platelets	+	+	+
Mixed vesicles	0	+	+
PLEP	0	0	+
Platelet 18:2 content (mol%)	8.3 ±0.5	9.3 ± 0.4	11.9 ± 0.7
Increase in 18:2 content	–	12 ± 5%	43 ± 8%
Platelet phospholipid content (nmol)	0.58 ± 0.02	0.60 ± 0.02	0.60 ± 0.02
Platelet ^{32}P cpm	–	9400 ± 1700	20800 ± 3800
Phosphatidylcholine adsorption and/or endocytosis (nmol) = b	–	11.0	11.0
Exchange phosphatidylcholine (nmol) = c	–	18.0	51.0
Platelet membrane 18:2 content (nmol) = d	96.0	113.0 (+18%)	147.0 (+53%)

serotonin. In all cases, platelets were able to release more than 85% of the [^{14}C]serotonin incorporated after incubation. On the other hand, the platelet aggregation is inhibited by incubation with mixed vesicles with or without phospholipid-exchange protein.

Physical parameters of the modified platelets

Fig. 2 displays the Arrhenius plots of platelet membrane microviscosity calculated from the DPH fluorescence anisotropy and from the fluorescence lifetime. No significant difference appears between the control and the dilinoleyl-treated platelets (Fig. 2A) for the microviscosity at 37°C (2.8 ± 0.2 P) and for the fusion activation energy (6.0 ± 0.3 kcal/mol). The values of the microviscosity of platelet membranes remain, for the control and for the dilinoleyl-treated cells, in close agreement with the values published by Shattil and Cooper [18] (2.84 ± 0.24 P for the platelet microviscosity at 37°C from 13 normal donors). The values obtained for ΔE are lower than the values found by Shattil [18] (E = 9.4 kcal/mol) but they stand in the range of 6–8 kcal/mol described for most types of cell-surface membrane [55].

Modification of the cholesterol content of the platelets cannot account for the observed deviation of E, since the liposomes have been equilibrated for their cholesterol content with the membrane. In Fig. 2B, in spite of a 30% increase

TABLE VII

PLATELET ADENYLATE CYCLASE ACTIVITIES

Adenylate cyclase activities are determined with lysates of control platelets and platelets incubated in presence of 5 mg phospholipid-exchange protein with mixed vesicles of either dilinoleylphosphatidylcholine/cholesterol (2 : 1) or 2-linoleylphosphatidylcholine/cholesterol (2 : 1). Basal activity, activity stimulated by NaF or prostaglandin E_1 are correlated with the unsaturation index of platelets (%) estimated as the amount of double bonds per 100 fatty acids. Activities are expressed as percentages of control activities. The control basal activity is 6.7 ± 0.3 pmol/min per mg protein. Statistical data are performed by Student's *t*-test; n.s., not significant.

Platelet enrichment: None		2-Linoleyl-phosphatidylcholine	Dilinoleyl-phosphatidylcholine
Percentage fatty acids			
16:0	27.3 ± 3.5	20.2 ± 2.3	25.3 ± 4.1
16:1	3.9 ± 0.5	5.0 ± 0.3	4.5 ± 0.3
18:0	19.9 ± 0.7	14.5 ± 1.0	17.3 ± 0.8
18:1	18.7 ± 1.0	22.3 ± 1.0	20.5 ± 0.4
18:2	4.4 ± 0.3	12.9 ± 0.5	10.9 ± 0.7
20:3	1.0 ± 0.1	1.5 ± 0.3	1.0 ± 0.2
20:4	22.5 ± 1.4	25.0 ± 1.5	19.6 ± 0.1
Unsaturation index (%)	123 ± 8	158 ± 8 ($P < 0.001$)	128 ± 5 (n.s.)
Adenylate cyclase activity			
Basal	100 ± 12	138 ± 2 ($P < 0.005$)	101 ± 10 (n.s.)
$1 \cdot 10^{-2}$ M NaF	100 ± 10	129 ± 8 ($P < 0.05$)	110 ± 5 (< 0.10)
$1 \cdot 10^{-6}$ M Prostaglandin E_1	100 ± 7	123 ± 13 ($P < 0.05$)	97 ± 6 (n.s.)

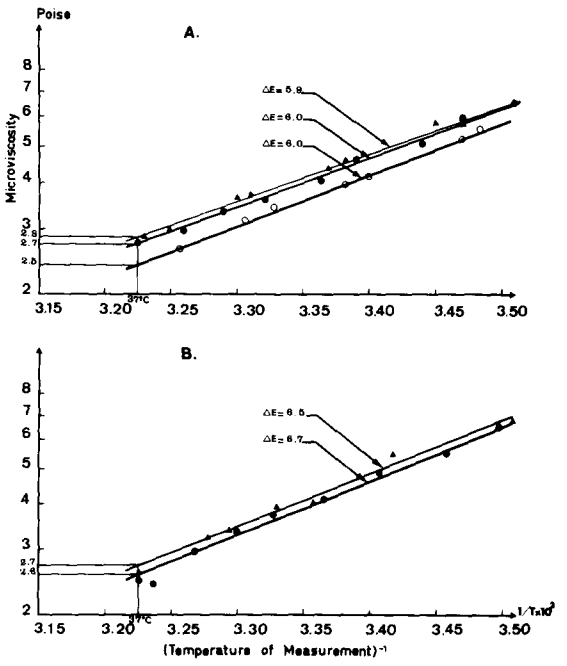

Fig. 2. Arrhenius plots of the platelets microviscosity. The microviscosity is calculated from the fluorescence anisotropy of diphenylhexatriene and from the fluorescence lifetime. Experiments are displayed with their controls after the enrichment with dilinoleylphosphatidylcholine (A) or with 2-linoleylphosphatidylcholine (B). The fusion activation energy (E as kcal/mol per K) and the microviscosity at 37°C (as P) are computed and displayed for each curve (1 S.D. for $E = \pm 0.3$ kcal/mol per K; 1 S.D. for η at 37°C = ±0.2 P). A: ○, 10^9 platelets + 2.8 mg phospholipid-exchange protein (PLEP); ●, $1 \cdot 10^9$ platelets + dilinoleylphosphatidylcholine vesicles (1 mol); ▲, $1 \cdot 10^9$ platelets + 2.8 mg phospholipid-exchange protein + dilinoleylphosphatidylcholine vesicles. B: ●, $1 \cdot 10^9$ platelets + 2-linoleylphosphatidylcholine vesicles (1 μmol); ▲, $1 \cdot 10^9$ platelets + 5 mg phospholipid-exchange protein + 2-linoleylphosphatidylcholine vesicles (1 μmol).

in the unsaturation index of the 2-linoleyl-treated platelets, no significant effect on the microviscosity is noticeable with regard to the control platelets. This is in agreement with personal unpublished results on the overestimation of the microviscosity modulated by the double-bond content of the membranes (liposomes or biomembranes).

Discussion

In order to study the possible changes in platelet functions induced by manipulation of acyl chain

composition of membrane phospholipids we have first used the physiological partners of platelets for phospholipid exchange in blood stream, i.e., plasma lipoproteins. When phospholipids are introduced into plasma as vesicles they are immediately taken up by the HDL_3 fraction [48]. Then there is immediately an exchange of the phospholipids between the different classes of plasma lipoproteins [56] which are also taken up by various tissues or cells [25,48].

In this study, the washed platelets are incubated in buffer with DLPC-loaded HDL. Despite the fact that such HDL exhibits a modified density and electrophoretic pattern, it is nevertheless a stable, well-defined lipoprotein structure [48,49]. Unfortunately, under our experimental conditions the contamination of the platelets by adsorbed vesicles through HDL-plasma membrane nonspecific interactions and/or endocytosis is of the same order as the exchange (Table II). Thus the incubation of washed platelets with loaded HDL does not appear to be a good tool for studying linoleyl-enriched platelet membranes.

In an attempt to obtain better control of exchange, we used unilamellar vesicles as donor membrane and rat liver phospholipid-exchange protein to catalyse the exchange. Some authors have shown that the rat liver phospholipid-exchange protein is not specific for phosphatidylcholines (see Ref. 51 for review). Under our conditions, phosphatidylcholine is the only exchangeable phospholipid. But the phospholipid-exchange protein preparation used before column chromatography is also able to stimulate cholesterol-ester exchange (Tables I and III and Fig. 1).

More recently it has been assessed that bovine liver phospholipid-exchange protein is also able to induce a net transfer of phospholipid from phosphatidylcholine liposomes to model membranes (phosphatidylethanolamine-phosphatidic acid, 4 : 1) [57]. Crain and Zilversmit [58] have described a new protein for this purpose: 'the nonspecific phospholipid transfer protein from bovine liver' [58] which can also catalyse the exchange process. This protein appears able to refill delipidated lipoprotein from phospholipid vesicles [58]. But the transfer activity is negligible with regard to the exchange activity when the two proteins act simultaneously [57]. This allows us to consider that under our experimental conditions only the exchange activity can be taken into account. This was confirmed by lipid phosphorus measurements: the platelet phospholipid levels remain constant after incubation with or without phospholipid-exchange protein (0.60 ± 0.02 μmol per 10^9 platelets).

When platelets were incubated with 2-linoleylphosphatidylcholine-cholesterol (2 : 1) vesicles, 17.0 nmol phosphatidylcholine are exchanged between $1 \cdot 10^9$ platelets and the vesicles (1 μmol phosphatidylcholine) in 1 h (Table VII). These results are in agreement with values of spontaneous exchange between platelets and HDL in platelet-rich plasma which reached 5 nmol of 2-[1-^{14}C]linoleylphosphatidylcholine per 10^9 platelets per h as we have previously established [2]. As linoleylphosphatidylcholine accounts for one-third of the human plasma phosphatidylcholine the whole exchange of phosphatidylcholine between the plasma lipoprotein and platelets is in the same range as the spontaneous exchange between platelets and mixed vesicles (15 nmol/10^9 platelets per h). Thus we cannot conclude whether HDL stimulate spontaneous exchange between membranes and plasma phospholipids or (according to Zilversmit's work [33]) not.

The addition of phospholipid-exchange protein (5 mg) stimulated 3-fold the exchange of 2-linoleylphosphatidylcholine (51 nmol/10^9 platelets per h), whereas adsorption and/or endocytosis are unaffected (Table VII).

In previous work we [21,51] and others [24] have shown that in 1 h the exchange affects mainly the outer leaflet of the plasma membrane. 60% of total platelet phospholipids are located in the plasma membrane with an assymmetrical localization [61]. In $1 \cdot 10^9$ platelets there are 580 nmol phospholipids and in the plasma membrane, 350 nmol. Phosphatidylcholine represents 34% of the platelet plasma membrane phospholipid [60]: that is to say, 119 nmol. 53 nmol are in the external leaflet, according to Chap et al. [61]. Incidentally, the exchange process which is measured by ^{32}P exchange (51 nmol/h, Table VII) affects nearly all the external phosphatidylcholine. The most part of the exchanged phosphatidylcholine stays in the external leaflet, since flip-flop is a very slow process in nonstimulated platelets as we have mea-

sured and reported in Ref. 51. But, according to the complex structure of platelet, we do not reach the same certainty as Van Meer et al. [26], who have tested the bovine liver phospholipid-exchange protein activity between liposomes and intact erythrocytes.

When platelets were incubated 1 h with DLPC-cholesterol vesicles (Fig. 1) we observed a 5% increase in the total linoleic acid level of platelets (8.1 mol% against 7.7), i.e., approx. 5 nmol linoleic acid incorporated in $1 \cdot 10^9$ platelets (i.e., 2.5 nmol DLPC). With phospholipid-exchange protein (5 mg) the level reaches 10.7 mol% (an approx. 30% increase). If we consider that there are 2 mol linoleic acid per mol phosphatidylcholine, one can assess that the DLPC does not behave as an efficient substrate for the exchange process. This may be explained by an effect of the dienoyl fatty acid in the 1-position of the glycerol backbone and a subsequent countereffect on the ability of molecules to pack together [62].

Before studying eventual changes in the functions of linoleyl-enriched platelets, we controlled viability of platelets during incubation by serotonin release and platelet aggregation. In each case after the incubation time, the platelets were able to release more than 85% of the [^{14}C]serotonin incorporated during a short preincubation. Platelet aggregability is altered by incubation in the presence or in the absence of phospholipid-exchange protein. It has already been evidence in this laboratory that the vesicles possess an inhibitory effect on platelet aggregation [64].

Measurements of the platelet adenylate cyclase activity assess that phospholipid-exchange protein alone has no effect on basal or stimulated activity. This agrees with the fact that phospholipid-exchange protein has a minimal perturbing effect on the membrane structure [65]. From Table VII and Fig. 2, one can see that neither basal, prostaglandin E_1-stimulated adenylate cyclase activity nor the physical parameters of platelets membranes are modified in DLPC-enriched platelets with regard to controls. Although the linoleic acid level in platelets is increased during incubation, we notice that the unsaturation index of platelets is not increased in the same period. This can be explained by the relative decreases in the other unsaturated species, especially those containing arachidonic acid which are localized on the inner leaflet [66]. It may result from a partial activation of platelets during manipulations with hydrolysis of arachidonic acid from the inner leaflet phospholipids [67]. On the other hand, when platelets are enriched in linoleic acid from 2-linoleylphosphatidylcholine we observe a significant increase in the unsaturation index of platelets (123 ± 8 to 158 ± 8) but the increase in linoleic acid accounts for only a part of the unsaturation index. At the same time, the basal activity of adenylate cyclase as well as the activities stimulated by $1 \cdot 10^{-2}$ M fluoride or by $1 \cdot 10^{-6}$ M prostaglandin E_1 are significantly increased.

In agreement with the results reported here, Engelhard et al. [9] have established a positive correlation between the adenylate cyclase activity stimulated by prostaglandin E_1 and the unsaturation index of mouse LM cell membranes by manipulating their fatty acid composition [9]. Neither these data nor ours could establish whether this interaction results from specific structural effect of phospholipid or from a change in the physical properties of the membrane. Neither membrane fluidity nor fusion activation energy measured by DPH fluorescence polarization is modified by linoleyl enrichment (Fig. 2). Nevertheless, values obtained with DPH fluorescence polarization represent an average microviscosity or activation energy of the whole platelet membranes. Lipid domains of different fluidity and asymmetry of fluidity between bilayer halves of plasma cell membranes have been described by several workers [68,69]. Therefore, we can overlook local changes in physical parameters in membranes, despite their potential importance in regulating membrane functions.

The increase in adenylate cyclase activities through 2-linoleyl enrichment is not due to contamination by linoleylphosphatidylcholine/cholesterol vesicles (adsorption and/or endocytosis), since activity of platelets incubated alone with vesicles is unchanged with regard to control. Because flip-flop is very slow, the enrichment in 2-linoleylphosphatidylcholine affects, on the whole, only the outer leaf of the platelet membrane, whereas the catalytic unit of adenylate cyclase is localized on the inner leaf [70]. Uncertainties concerning membrane topology do not permit further discussion of these data.

In this work, we evidence a positive correlation between the 2-linoleylphosphatidylcholine enrichment and the adenylate cyclase activity in the platelet membranes. This favors the assumption of some specific action of 2-linoleylphosphatidylcholine as an inducer of the effect of the linoleyl moiety on adenylate cyclase which has already been evidenced by Orly and Schramm [59] in the case of turkey goose erythrocytes and by Colard et al. [63] for rat hepatocyte membranes.

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