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Use of Phospholipid Transfer Protein as a Probe to Study the Lipid Dynamics and Alkaline Phosphatase Activity in the Brush Border Membrane of Human Term Placenta

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Incubation of placental brush border membrane (BBM) along with sonicated vesicles of exogenous lipids (egg yolk PC) in the presence of phospholipid-transfer protein (PL-TP) showed a decrease in the alkaline phosphatase activity due to the change in the membrane micro-environment, such as fluidity. Effect of substrate concentration was tested by *Lineweaver–Burk* plot, which showed decreased V_{max} and K_M. The effect of temperature was probed by the *Arrhenius* plot, which showed no change in transition temperature, but a decline in the energy of activation both below and above the transition temperature. The protein-catalyzed transfer of phospholipid from the donor unilamellar vesicles resulted in a substantial increase in the BBM phospholipid and a net decrease in cholesterol/phospholipid molar ratio. The change in membrane fluidity was assessed by translational as well as rotational diffusion of membrane extrinsic fluorescent probes, pyrene and diphenyl-hexatriene. An increased lateral mobility was recorded by the increased pyrene excimer formation. A decrease in fluorescent polarization of diphenyl-hexatriene was observed, which led to the decrease in fluorescence anisotropy and order parameter, and therefore, an increase in membrane fluidity (rotational diffusion). Mean anisotropy parameter was also decreased in the presence of PL-TP. Thus, the placental BBM alkaline phosphatase activity showed a distinct lipid dependence which may have important physiological consequences.

Introduction. – The plasma membrane of placental syncytial trophoblast cells contains two distinct regions, the microvillus or brush border membrane (BBM), and the basal cell membranes, which are distinguished morphologically, biochemically, and functionally [1–4]. The BBM forms the effective barrier separating the maternal blood from the fetal circulation and is important in the exchange of nutrients, hormones, waste products, and other molecules between the mother and the fetus [5]. The membrane is also richly endowed with alkaline phosphatase activity, which is believed to be intimately associated with the phosphate transport process [6]. Functional dependence of the membrane-bound enzymes on lipid micro-environment has been a subject of continued research interest in this laboratory [7–9]. The phospholipid-transfer proteins (PL-TPs) have been used as excellent membrane probes in modifying the physicochemical properties and functional dependence of the membrane protein [10–12]. In earlier studies, the lipid-transfer proteins have been effectively used in membrane modification and functional studies in myelin and brain microsomes [13–15], intestinal BBM [16][17], and lung surfactant [18][19]. In the present study, the molecular dynamics of the lipid bilayer and its interaction with alkaline phosphatase activity has been investigated in the BBM of human term placenta using the technique

of protein-mediated lipid transfer, and the translational and rotational diffusion characteristics of the membrane.

Results and Discussion. – *Effects of PL-TP on the Placental BBM Alkaline Phosphatase*. Human term placental BBM has been isolated and further modulated in the presence of liposomes and PL-TP. The purity of the isolated membranes was checked, and an enrichment of 10–15-folds was found in terms of the alkaline phosphatase activity when compared with the homogenates, which corresponds well with the report of other workers [20]. The importance of the enzyme in BBM lies in a specific phosphate transport pathway at the physiological pH [21][22].

Table 1 shows that incubation of unilamellar vesicles (ULVs) with the BBM in the presence of PL-TP resulted in a significant loss of activity of alkaline phosphatase. ULVs were composed of two types of lipids; one with the extracted lipids of BBM (endogenous) while the other one composed of egg yolk PC and cholesterol (Ch; exogenous) in different molar ratio. The maximum enzyme inhibition was seen when incubated with the PC liposomes.

Table 1. *The Alkaline Phosphatase Activity of Human Term Placental BBM in the Presence of Sonicated Vesicles of Egg-yolk PC and Cholesterol Having Different Mole Ratios and Lipids Extracted from the Placental BBM in the Presence and Absence of Lipid-Transfer Protein*

Membrane	Alkaline phosphatase [mmol/mg protein][a])	
	Transfer Protein	
	without	with
BBM	1.390 ± 0.091	1.444 ± 0.138
BBM + ULVs:		
PC	1.302 ± 0.064	1.202 ± 0.080
PC/Ch (1 : 0.1)	1.814 ± 0.174	1.424 ± 0.096[b])
PC/Ch (1 : 1)	1.203 ± 0.051	0.927 ± 0.012[c])
PC/Ch (1 : 10)	1.037 ± 0.026	0.877 ± 0.012
PE	1.344 ± 0.147	0.893 ± 0.113[b])
PI	1.588 ± 0.095	1.014 ± 0.131[d])
PS	1.396 ± 0.074	1.148 ± 0.048[b])
GL	1.371 ± 0.056	0.977 ± 0.096[d])
PC	1.561 ± 0.056	0.841 ± 0.063[c])
SM	1.143 ± 0.114	0.784 ± 0.109[a])

[a]) Values represent mean ± S.D. for three independent observations. [b]) $p < 0.05$ when compared with values having no transfer protein. [c]) $p < 0.001$ when compared with values having no transfer protein. [d]) $p < 0.01$ when compared with values having no transfer protein.

The *Figure* demonstrates the dependence of enzymatic hydrolysis on the increasing substrate concentration ranging from 1–7 mM, which is also evident in the resultant *Lineweaver–Burk* plot of the double reciprocals of substrate concentration and enzyme velocity. The K_m and V_{max} values, which were obtained from the plot, were decreased in the presence of PL-TP. These kinetic parameters were further measured at different temperatures (4–45°), and the values presented in the form *Arrhenius* expression as a relationship of reciprocal of temperature and the $\log_{10} K_m$ or V_{max}. A

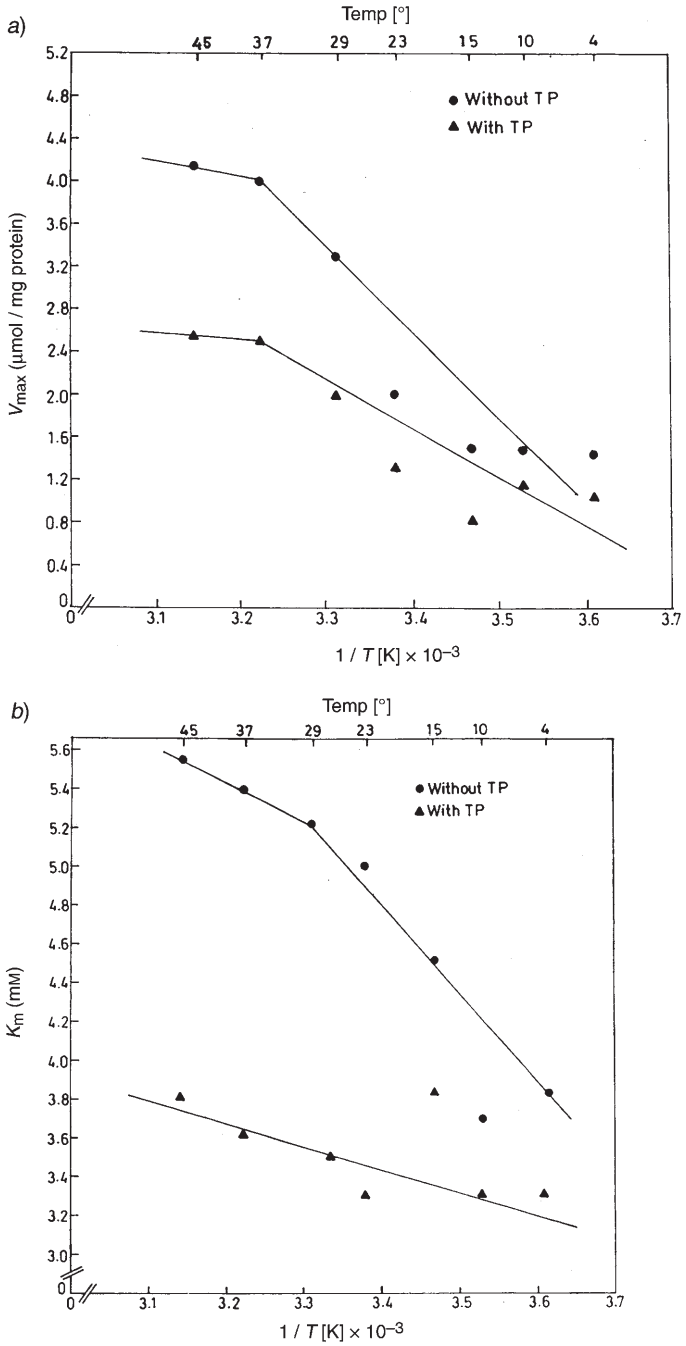

Figure. a) Arrhenius *plot of microvillus membrane alkaline phosphatase constructed from the kinetic parameter,* V_{max} [µmol/mg protein] vs. *the reciprocal of the absolute temp* [K] *in the presence* (▲) *and absence* (●) *of phospholipid-transfer protein.* b) Arrhenius *plot of microvillus membrane alkaline phosphatase constructed from the kinetic parameter,* K_m [mM] vs. *the reciprocal of the absolute temperature* [K] *in the presence* (▲) *and absence* (●) *of phospholipid-transfer protein.*

non-linear *Arrhenius* plot of alkaline phosphatase both in the native and modified BBM curves was observed with biphasic slopes. The apparent break point was corresponding to the transition temperature of the enzyme and may relate to the lipid phase transition of the membranes. The enzyme showed a characteristic break point in V_{max} at 37.4° in the presence and absence of PL-TP, respectively, which, however, was not visible when K_m was plotted. A break point was seen only in the membrane without PL-TP with the transition temperature recorded at 290°, while the membranes modified with PL-TP showed a continuous line. Energy of activation was calculated from the slope using the equation $E_a = -\text{slope} \times 2.303\ RT$ and as can be seen from the *Table 2*, the activation energy decreased considerably in the presence of PL-TP.

Table 2. *Thermodynamic Parameters of the Alkaline Phosphatase at Optimum Substrate Concentration in the Placental BBM Incubated along with the Sonicated Vesicles of Egg Yolk PC and Cholesterol in a 1 : 1 Mole Ratio*[a])

Membrane	Transition Temp. (T_c) [°]	Energy of activation (E_a) [cal/mol]	
		below T_c	above T_c
BBM + PC/Ch Lipid vesicles	31.4	1232	593
BBM + PC/Ch Lipid vesicles + Lipid transfer protein	25.5	1033	415

[a]) The data is based on the average of two independent experiments, assayed in duplicates.

Effect of PL-TP on Membrane Lipid Composition in Placental BBM. BBM Lipids were extracted, and the cholesterol and phospholipid contents were measured, when incubated along with or without the unilamellar liposomes and PL-TP. The cholesterol/PL molar ratio was found to vary over a range of 0.136 to 0.378 from a normal value of 0.158 when the membranes were incubated with endogenous liposomes and the PL-TP (*Table 3*). In the absence of PL-TP, the ratio varied from 0.158 to 0.429. A decrease was observed in the case of exogenous liposomes compared of egg yolk PC and cholesterol in different molar ratio where the cholesterol/PL molar ratio varied from 0.184 to 0.285 in the absence of PLTP, and 0.095 to 0.173 in the presence of the protein, respectively. Similar decrease in fructose-6-phosphate phosphohydrolase activity was reported in the presence of non-specific lipid-transfer protein in rat liver microsomal membranes [23]. The decrease in the activity of alkaline phosphatase by *ca.* 20–30% was observed in the presence of non-specific lipid-transfer protein in rat intestinal microvillus membranes [12]. The lipid-transfer proteins are potentially powerful tools in the elucidation of the phospholipid dependence of enzymatic activity. It has been used here to alter the lipid composition of the placental BBM without the denaturing effects of other agents like the phospholipases, or of disintegration/reconstitution procedures, and possibly without affecting the organization of the enzyme proteins.

Effect of PL-TP on Pyrene Excimer Formation in Placental BBM. The relationship between the cholesterol/PL molar ratio and pyrene excimer/monomer (E/M) ratio in the placental BBM in the presence and absence of PL-TP has been shown in *Table 4*. The increased value of E/M led to a decrease in the microviscosity (reciprocal of fluidity), which was presented as $E/M = [\text{pyrene}] \times KT/\eta$, where η represents the microviscosity. The incubation of the membrane with the PL-TP led to an increase in

Table 3. *Cholesterol/phospholipid Molar Ratio of Human Term Placental BBM alone as well as along with Exogenous Lipid Vesicles in the Presence and Absence of Lipid Transfer Protein*[a])

Membrane	Ch/PL (Molar ratio)	
	Transfer protein	
	without	with
BBM	0.158	0.158
BBM + ULVs:		
PC	0.256	0.095
PC/Ch (1 : 0.1)	0.261	0.147
PC/Ch (1 : 1)	0.285	0.146
PC/Ch (1 : 10)	0.234	0.173
PE	0.378	0.147
PI	0.404	0.378
PS	0.287	0.205
GL	0.178	0.136
PC	0.429	0.202
SM	0.329	0.221

[a]) The data is based on the average of two independent experiments, assayed in duplicates.

Table 4. *Excimer Formation and the Resultant Membrane Lipid Microviscosity*[a]) *in Human Term Placental BBM alone as well as along with the Vesicles of Exogenous Lipids in Different Mole Ratios with and without Lipid Transfer Protein*[b])

Membrane	Excimer/Monomer (E/M)		Microviscosity (Poise)	
	Transfer protein		Transfer protein	
	without	with	without	with
BBM	0.27 ± 0.01	0.28 ± 0.01	6.98 ± 0.25	6.72 ± 0.24
BBM + ULVs:				
PC	0.30 ± 0.02	0.32 ± 0.01	6.28 ± 0.48	5.94 ± 0.18
PC/Ch (1 : 0.1)	0.42 ± 0.04	0.68 ± 0.14	4.53 ± 0.43	2.81 ± 0.6[c])
PC/Ch (1 : 1)	0.26 ± 0.04	0.27 ± 0.01	7.24 ± 1.15	6.98 ± 0.25
PC/Ch (1 : 10)	0.25 ± 0.04	0.26 ± 0.05	7.53 ± 1.25	7.24 ± 0.39
PE	0.33 ± 0.05	0.26 ± 0.02	5.71 ± 0.86	7.24 ± 0.56
PI	n.d.[d])	n.d.[d])	n.d.[d])	n.d.[d])
PS	0.48 ± 0.025	0.80 ± 0.12	3.93 ± 0.41	2.38 ± 0.59[c])
GL	0.82 ± 0.12	1.09 ± 0.19	2.30 ± 0.57	1.74 ± 0.18
PC	0.75 ± 0.11	0.76 ± 0.06	2.55 ± 0.37	2.48 ± 0.19
SM	0.61 ± 0.02	0.65 ± 0.04	3.08 ± 0.10	2.89 ± 0.18

[a]) Microviscosity (η) was calculated from the relationship E/M = [Pyrene]Tk/η where T = absolute temperature and k is the *Boltzman* constant (1.38×10^{-23} J/K). [b]) Values represent mean ± S.D. of three independent observations. [c]) $p < 0.01$, when compared with values having no transfer protein. [d]) n.d.: 'not detected'.

the membrane fluidity. There was a significant change in E/M ratio in the case of sonicated vesicles having exogenous lipids of cholesterol/PL molar ratio of 1 : 0.1. Similarly, a significant change was found in case of the vesicles of endogenous lipids of

PS. In general, the efficiency of pyrene excimer formation was increased by lipid incorporation in BBM and resulted in a decrease in the microviscosity. However, with the increasing content of cholesterol in the exogenous lipid vesicles, the efficiency to form pyrene excimer decreases and resulted in an increased microviscosity. Pyrene excimer formation which quantitates the translational mobility has been used as a fluidity probe in many model and biological systems [24]. The apparent microviscosity in biological membrane ranges from 1–10 poise at room temperature, and, therefore, the value recorded in the present membrane system is well within such limit.

Effect of PL-TP in the Fluorescence Polarization of DPH in the BBM of Placenta. There was a decrease in fluorescence polarization and anisotropy of DPH in the presence of PL-TP in the placental BBM when incubated with the sonicated liposomes of endogenous or exogenous lipids (*Table 5*). The slow decaying component r_∞, which is the static component of the steady-state fluorescence anisotropy (r_s), was also decreased. This led to a lower order parameter (S_{DPH}) in the presence of PL-TP. The alterations in the values of r_s, r_∞, and S_{DPH} were found to be closely correlated with the changes in the cholesterol content of the exogenous lipid vesicles. The membrane order parameter varied over a range of 0.206 to 0.583 from a normal value of 0.574 of native BBM with PL-TP in case of exogenous liposomes, which, however, varied from 0.269 to 0.612 in the absence of PL-TP with a normal value of 0.574. Similarly, the S_{DPH} value varied over a range of 0.224 to 0.274 in case of endogenous liposomes with PL-TP as compared to 0.206 to 0.224 without PL-TP. A significant change was also found when the membrane was incubated with PS liposomes prepared from the BBM endogenous lipids. Fluorescence polarization is a measure of the relative rotational mobility of a fluorescent probe incorporated into a membrane and has been used to study from the simple liposomes to the complex cell membranes [24]. Membranes in which there is high rotational ability of the fluorescent probe have lower fluorescence polarization and, therefore, fluidity [25]. In the present study, the decreased fluorescence polarization in the presence of PL-TP in both the cases of exogenous and endogenous lipid vesicles resulted in more fluidity. This is substantiated by decrement of the fluorescence anisotropy values, which is particularly pronounced in case of membrane modifications with PC vesicles. From these values, order parameters have been derived which show a decrement. As lipid fluidity is reciprocal to the structural order parameter (S_{DPH}) [26], the lipid fluidity in the placental BBM is increased with PL-TP in the present study, and thus led to the modulation of the alkaline phosphatase activity.

Conclusions. – Phospholipid transport protein, when used as a membrane probe, was found to modulate the alkaline phosphatase activity in the placental BBM and a change in the membrane fluidity. Also, the BBM can be effectively used as an experimental tool for the insertion of exogenous lipids for the donor membrane modification. The BBM is richly endowed with alkaline phosphatase, which is believed to be intensely associated with phosphate transport process. Therefore, such information on the membrane modification, fluidity, and the change in enzymatic activity can be extremely useful in the elucidation of the molecular mechanism of nutrient transport between the mother and foetus.

Table 5. *Fluorescence Polarization* (P_{DPH}), *Fluorescence Anisotropy* (r_s), *Lipid Order Parameter* (S_{DPH}), *and Mean Anisotropy Parameter, in Human Term Placental BBM alone as well as along with the Vesicles of Exogenous Lipids in Different Mole Ratios, in the Presence and Absence of Lipid Transfer Protein*[a])

Membrane	Fluorescence polarization (P_{DPH})		Fluorescence anisotropy (r_s)		Slow decaying component (r_∞)		Order parameter (S_{DPH})		Mean anisotropy parameter $[(r_0/r_s)^{-1}]^{-1}$	
	Transfer protein		Transfer protein		Transfer protein		Transfer protein		Transfer protein	
	without	with	without	with	without	with	without	with	without	with
BBM	0.240	0.249	0.174	0.179	0.132	0.138	0.574	0.587	0.770	0.810
BBM + ULVs										
PC	0.115	0.092	0.084	0.063	0.029	0.017	0.269	0.206	0.266	0.186
PC/Ch (1 : 0.1)	0.208	0.180	0.148	0.127	0.097	0.069	0.492	0.415	0.587	0.465
PC/Ch (1 : 1)	0.250	0.237	0.182	0.171	0.142	0.128	0.596	0.565	0.835	0.750
PC/Ch (1 : 10)	0.257	0.244	0.187	0.177	0.15	0.136	0.612	0.583	0.877	0.793
PE	0.110	0.065	0.076	0.049	0.022	0.008	0.234	0.141	0.234	0.139
PI	n.d.[b])	n.d.[b])	n.d.[b])	n.d.[b])	n.d.[b])	n.d.[b])	n.d.[b])	n.d.[b])	n.d.[b])	n.d.[b])
PS	0.133	0.092	0.091	0.063	0.030	0.017	0.274	0.206	0.294	0.186
GL	0.101	0.097	0.070	0.067	0.020	0.018	0.224	0.212	0.212	0.201
PC	0.113	0.107	0.078	0.074	0.023	0.021	0.240	0.229	0.242	0.226
SM	0.120	0.100	0.083	0.070	0.028	0.020	0.265	0.224	0.262	0.212

[a]) The data is based on the average of two independent experiments, assayed in duplicates. [b]) n.d.: not detected

Experimental Part

Following methods have been described in great detail in earlier publications: preparation of human term placental BBM and characterization [1–4]; isolation of phospholipid transfer protein and characterization [16–19]; analysis of membrane lipids [27][28]; preparation of liposomes [7][8]; lipid transfer assay [7][8][16–19], and the analytical methods [19]; membrane modification by lipid transfer [8][19] and membrane microviscosity measurement by pyrene fluorescence excimer (dimer) formation [18]. The enzyme alkaline phosphatase (E. C. 3.1.3.1) was routinely assayed in the placental BBM preparation by adopting the method of *Bergmeyer* [29] as outlined in [2]. The assay was performed in triplicates at 37° for 15 min at a protein concentration which gave an activity within the linear range for the assay. Also, the substrate (4-nitrophenyl phosphate) concentration was used in all the experiments so as to ensure linear kinetics (saturating concentration), and not more than 5–10% of the substrate was utilized within the assay period. The substrate was hydrolyzed at an alkaline pH 10.5 to yield a yellow colored product, 4-nitrophenol, which was measured at 420 nm. A suitable range of standards (4-nitrophenol) and the blank were also run simultaneously in which all other reagents were added except for the enzyme which was added after the termination of the incubation.

Membrane Fluorescence Polarization Studies of 1,6-Diphenylhexa-1,3,5-triene (DPH) as Membrane Extrinsic Probe. The membrane fluidity was assessed in the present study by steady state fluorescence polarization technique using the hydrocarbon fluorophore DPH according to the method of *Lackowicz* [20]. The steady-state fluorescence anisotropy (r_s) and polarization (P) were determined by the emission intensities through an analyzer, oriented parallel ($I_\parallel$) and perpendicular ($I_\perp$) to the direction of polarization of the excitation light as mentioned by *Blitterswijk et al.* [26] as follows:

$$r_s = \frac{I_\parallel - I_\perp}{I_\parallel + 2I_\perp}, \quad P = \frac{I_\parallel - I_\perp}{I_\parallel + I_\perp}$$

Membrane suspension along with sonicated lipid vesicles (natural and synthetic) in the presence and absence of PL-TP were prepared (0.5 mg protein/500 µl). The above suspension (50 µl) was mixed with an aliquot of probe dispersion (DPH; 25 mM in abs. EtOH), and incubated for 30 min in a water bath at

37° with continuous shaking. The total volume was made 3 ml with a liposomal buffer (5 mM HEPES, 10 mM NaCl, pH 7.4). Also, the membrane samples without DPH were prepared, and the corrections for light scattering were made with appropriate filter.

The fluorescence-intensity measurements were conducted in a *UVIKON SFM 25* (*Kontron Instruments*) fluorescence spectrophotometer with polarizer/analyzer attachment. The excitation wavelength used was 352 nm to measure the emission fluorescence at 435 nm with the appropriate cut-off filter. The steady-state fluorescence anisotropy, r_s, is resolved into a fast-decaying component r_f and an infinitely slow-decaying component which is called the static component r_∞. The later contribution, which predominates in biological membranes, is exclusively determined by the degree of molecular packing (order) in the apical regions of the membranes; r_∞ is proportional to the square of the lipid order parameter (S):

$$s^2 = \frac{r_\infty}{r_0}, \quad 0 \leq s \leq 1$$

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