Influence of Membrane Phospholipid Composition and Structural Organization on Spontaneous Lipid Transfer Between Membranes

R. Pankov[1], T. Markovska[2], P. Antonov[3], L. Ivanova[4] and A. Momchilova[2]

[1] *Biological Faculty, Sofia University, Sofia, Bulgaria*
[2] *Institute of Biophysics, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria*
[3] *Department of Physics and Biophysics, Medical University, 1431 Sofia, Bulgaria*
[4] *Cytos Biotechnology AG, Wagistrasse 25, Postfach, CH–8952 Zurich, Switzerland*

Abstract. Investigations were carried out on the influence of phospholipid composition of model membranes on the processes of spontaneous lipid transfer between membranes. Acceptor vesicles were prepared from phospholipids extracted from plasma membranes of control and *ras*-transformed fibroblasts. Acceptor model membranes with manipulated levels of phosphatidylethanolamine (PE), sphingomyelin and phosphatidic acid were also used in the studies. Donor vesicles were prepared of phosphatidylcholine (PC) and contained two fluorescent lipid analogues, NBD-PC and N-Rh-PE, at a self-quenching concentration. Lipid transfer rate was assessed by measuring the increase of fluorescence in acceptor membranes due to transfer of fluorescent lipid analogues from quenched donor to unquenched acceptor vesicles. The results showed that spontaneous NBD-PC transfer increased upon fluidization of acceptor vesicles. In addition, elevation of PE concentration in model membranes was also accompanied by an increase of lipid transfer to all series of acceptor vesicles. The results are discussed with respect to the role of lipid composition and structural order of cellular plasma membranes in the processes of spontaneous lipid exchange between membrane bilayers.

Key words: Spontaneous lipid transfer — Model membranes — Lipid structural organization

Introduction

Spontaneous transfer of lipid molecules between membrane bilayers is a ubiquitous process of significant biological importance (Nichols and Pagano 1981; Pagano and Sleight 1985). Although the mechanism of monomer lipid transfer between lipid

Correspondence to: Roumen Pankov, Department of Cytology, Histology and Embryology, Biological Faculty, Sofia University, Dragan Tzankov str. 8, 1164 Sofia, Bulgaria
E-mail: rpankov@biofac.uni-sofia.bg

bilayers *in vivo* is not yet clear, there is extensive evidence that lipid molecules can undergo spontaneous transfer between membranes (Struck and Pagano 1980; Nichols and Pagano 1981).

It is well known that phospholipid composition of plasma membranes plays a significant role in cell-to-cell interactions and lipid exchange between cells and blood plasma (Struck and Pagano 1980; Pagano and Sleight 1985). In this paper, we studied the influence of phospholipid composition of acceptor membranes derived from plasma membranes of two different cell lines – control and *ras*-transformed fibroblasts – on spontaneous transfer of lipids to these membranes. This experimental model was chosen because vesicles prepared from cell-derived lipids represent a much more relevant model of cellular plasma membranes compared to vesicles containing synthetic lipids. In addition, control and *ras*-transformed fibroblasts have some established differences in their lipid metabolism and composition (Momchilova et al. 1998, 1999), which makes them a convenient experimental model for comparison of obtained results.

In a previous study, we have observed elevated rate of fatty acid uptake in *ras*-transformed fibroblasts (Momchilova et al. 1999). Also, stimulated endocytosis has been reported in *ras*-transformed cells, which is related to the specific localization of p21 proteins in the cellular plasma membranes (Lacal and Carnero 1994). In the present studies, we made an attempt to shed some light on the processes of spontaneous exchange of lipids between plasma membranes of cells with different phospholipid composition. Such studies would help to better understand the mechanisms underlying the reported differences in the composition and behavior of plasma membrane lipids of control and transformed cells. Our investigations were focused especially on the role of lipid composition and structural order of membranes in spontaneous lipid transfer between them, and tried to extrapolate the obtained data to lipid exchange processes between living cells. For this purpose, acceptor vesicles containing membrane-derived lipids from the two cell lines (control and *ras*-transformed) were subjected to manipulations of levels of specific phospholipids. The content of two major lipid fractions – phosphatidylethanolamine (PE) and sphingomyelin (SM) – was manipulated, because they showed most significant differences between the two sets of acceptor vesicles. The level of phosphatidic acid (PA), which is a minor membrane lipid, but a potent participant in the *ras* signaling pathway (Lacal and Carnero 1994), was also altered in the acceptor vesicles. The results showed that all manipulations of acceptor vesicles lipids affected the spontaneous transfer of fluorescent phosphatidylcholine (PC). The role of membrane composition and structural organization is discussed with respect to the processes of lipid exchange and cell-to-cell interactions.

Materials and Methods

Materials

1-oleoyl-2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phosphocholine (NBD-PC) and N-(lissamine rhodamine B sulfonyl) dioleoyl pho-

sphatidylethanolamine (N-Rh-PE) were obtained from Avanti Polar Lipids (Alabaster, AL). Culture medium, antibiotic/antimycotic solution and serum were from GIBCO BRL Life Technologies. Phospholipase D, phospholipids, DPH, EDTA and other chemicals (except where otherwise stated) were from Sigma Chemicals Co. (St. Louis, MO).

Cell culture

NIH 3T3 fibroblasts transfected with activated human Ha-*ras* gene (Der 1986) and the parental cell line were a kind gift from Dr. Channing J. Der (University of North Carolina at Chapel Hill, Chapel Hill, NC). The cells were cultured as described elsewhere (Momchilova 1999). In brief, fibroblasts were grown in Dulbecco's modified Eagles medium containing 400 μg/ml G 418 (Geneticin), supplemented with 9% (v/v) fetal calf serum at 37°C in a humidified 5% CO_2 atmosphere. To obtain cell suspension, the monolayers were harvested by treatment with 0.05% trypsin and resuspended by pipetting in HEPES-buffered saline (in mmol/l): 136 NaCl, 2 KCl, 0.5 $MgCl_2$, 5 glucose, 10 HEPES (pH 7.4). Plasma membranes were isolated from control and *ras*-transformed cells as described elsewhere (Pankov et al. 2005). This procedure included loading of the post-nuclear supernatant on a discontinuous sucrose gradient and centrifugation at 100,000 $\times$ *g* for 2.5 h. Plasma membrane fraction was collected at a density of approximately 45% (w/v) and was suspended in ice-cold 100 mmol/l buffer and used immediately for lipid extraction as described below.

Preparation of lipid vesicles

Unilamellar donor vesicles were prepared by ethanol injection as described by Pagano et al. (1981). Briefly, the vesicles contained 98 mol% 1-palmitoyl-2-oleoyl-*sn*-glycerophosphocholine, 1 mol% C_6-NBD-PC, and 1 mol% of N-Rh-PE as non-exchangeable fluorescence resonance energy transfer acceptor NBD-lipids (Dao et al. 1991). Insertion of the fluorescent lipids only into the outer vesicle monolayer was achieved by adding the corresponding amounts of fluorescent lipids in ethanol to preformed unilamellar vesicles (1 mg of lipid/ml). The suspension thus obtained was dialyzed overnight against HEPES saline. The localization of NBD-PC exclusively in the outer vesicle monolayer was tested by assessing the total transfer of NBD-PC to a set of acceptor membranes. The acceptor vesicles containing plasma membrane phospholipids were prepared by ultrasonication under inert nitrogen atmosphere. Modulation of PE and SM levels was achieved by addition of the corresponding lipid quantity (70% of the quantity of PE or SM calculated *per* ml) to 1 ml of chloroform solution containing the total phospholipid extract from plasma membranes. Then, the extract was dried under nitrogen and small unilamellar vesicles were prepared by sonication.

Fluorescence measurements

Fluorescence anisotropy of DPH was used to estimate structural order of the membranes (Shinitzky and Barenholtz 1974). Steady-state anisotropy of DPH fluores-

cence r_s was estimated using the formula:

$$r_s = (I_{II} - I_{\perp})/(I_{II} + 2I_{\perp})$$

The lipid structural order parameter (S_{DPH}) was determined by an empirical method described by Van Blitterswijk et al. (1981). The value of S_{DPH} was calculated using the formula:

$$S_{DPH} = (r_{\infty}/r_o)^{1/2}$$

where r_o (0.395) is the initial anisotropy of fluorescence before any molecular motion takes place (Jahnig 1979). An empirical equation gives the relation between r_{∞} and r_s:

$$r_{\infty} = 4/3r_s - 0.1$$

(Van Bliterswijk et al. 1981). Steady-state fluorescence was measured at 530 nm (λ_{ex} = 470 nm) for NBD-PC and at 425 nm (λ_{ex} = 355 nm) for DPH using a Perkin Elmer 3000 fluorescence spectrometer.

Measurement of lipid transfer

The lipid transfer assay was performed as described by Dao et al. (1991) and McIntyre and Sleight (1991). Acceptor membranes containing a 100-fold excess of unlabeled lipids were incubated with donor vesicles for 20 min. Spontaneous transfer of labeled PC from donor to acceptor vesicles was accompanied by an increase in fluorescence due to dequenching of the fluorescent lipid (Dao et al. 1991). The lipid transfer was assessed using the equation:

$$F_{tr} = F_{eq} - F_i - F_{acc}$$

in which F_{tr} is the fluorescence intensity of NBD-PC molecules transferred from donor to acceptor membranes, F_{eq} is the fluorescence at equilibrium of donor and acceptor vesicles together, F_{acc} is the fluorescence of acceptor vesicles before addition of donor vesicles and F_i is the fluorescence of NBD-PC containing vesicles before being added to the incubation medium.

Lipid analysis

Extraction of plasma membrane phospholipids was performed with chloroform/methanol according to the method of Bligh and Dyer (1959). The organic phase obtained after extraction was concentrated and analyzed by thin layer chromatography. Phospholipid fractions were separated on silica gel G 60 plates (Merck) in a solvent system containing chloroform/methanol/isopropanol/triethylamine/0.25% KCl (30 : 9 : 25 : 18 : 6 v/v) (Touchstone et al. 1980). The phospholipid content of each fraction was determined by measuring inorganic phosphorus.

Other procedures

Treatment of acceptor membranes with phospholipase D was performed as described elsewhere (Pankov et al. 2005). Differences between means were analyzed by Student's *t*-test.

Results

Plasma membranes of control and *ras*-transformed fibroblasts were used as a source of total phospholipids. The phospholipid composition of acceptor membranes containing total lipids of native plasma membranes isolated from the two cell lines is shown in Fig. 1. Clearly, the membranes of control and *ras*-transformed fibroblasts differed in all analyzed lipid fractions. However, the most substantial differences between the two cell lines were observed for SM and PE, which were elevated in the transformed cells (Fig. 1). The rest of the lipid fractions were lower in *ras* cells, but these differences were less pronounced compared to those observed for PE and SM. The two different sets of acceptor vesicles prepared of cell-derived lipids from plasma membranes of either control or *ras*-transformed cells were incubated with donor vesicles containing the fluorescent lipid probes, NBD-PC and N-Rh-PE. The NBD-lipid analogues, although being transferred faster than their natural counterparts, provide very useful information on inter-membrane lipid transfer and are used widely in studies of lipid exchange processes. Lipid transfer rate was assessed by measuring the increase of fluorescence in acceptor membranes due to lipid transfer from the quenched donor to unquenched acceptor vesicles (Dao et

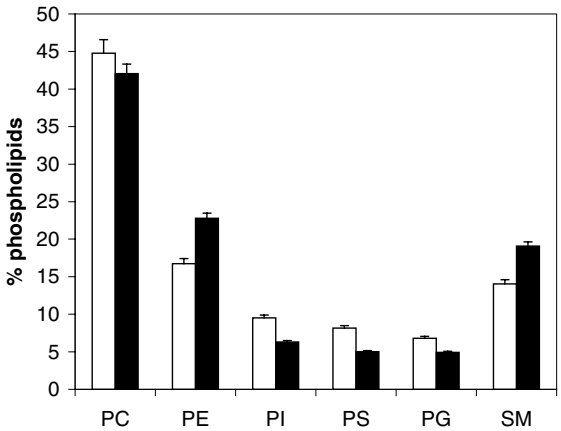

Figure 1. Phospholipid composition of acceptor vesicles containing lipids derived from plasma membranes of control (white columns) and *ras*-transformed (black columns) fibroblasts. Values are means ± SD from three determinations in three separate experiments. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PG, phosphatidylglycerol; SM, sphingomyelin.

Table 1. Transfer of NBD-PC from donor to acceptor model membranes

Manipulation of acceptor vesicles	F_{tr} in acceptor vesicles containing lipids from	
	Control cells	*ras* cells
None	53*	69*
PE	76**	89**
SM	41**	56**
Phospholipase D	64**	77**

F_{tr} represents the relative units of NBD-fluorescence transferred from donor to acceptor vesicles calculated as described in Materials and Methods. Values are means from three separate experiments performed in triplicates. * statistical significance was calculated using this value as reference; ** $p < 0.01$.

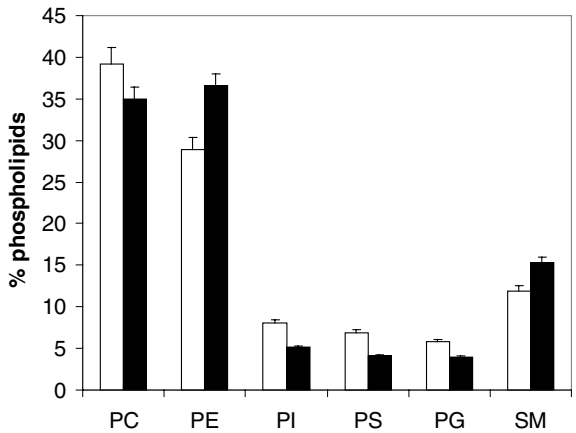

Figure 2. Phospholipid composition of PE-enriched acceptor vesicles containing lipids derived from plasma membranes of control (white columns) and *ras*-transformed (black columns) fibroblasts. Values are means ± SD from three determinations in three separate experiments. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PG, phosphatidylglycerol; SM, sphingomyelin.

al. 1991). Transfer effectiveness of the labeled PC molecules to acceptor vesicles is shown in Table 1. The results indicate that the transfer of fluorescent PC was higher towards acceptor vesicles containing *ras* cells phospholipids than to acceptor vesicles containing phospholipids of control fibroblasts.

In order to study the role of the differences in lipid composition of the two types of acceptor membranes, we selectively modified their lipid composition and analyzed the effect of the corresponding changes on lipid transfer. For this purpose, the levels of the two phospholipids that showed the most marked differences between

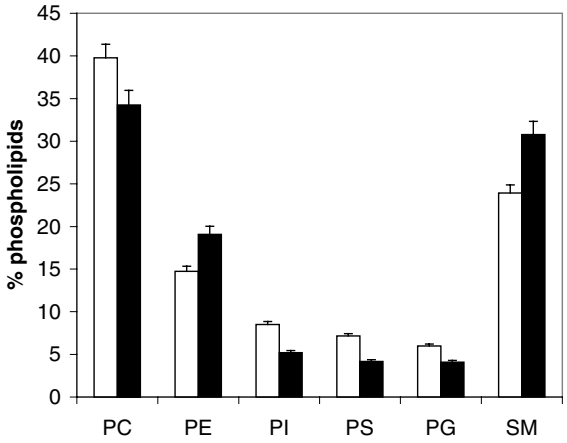

Figure 3. Phospholipid composition of SM-enriched acceptor vesicles containing lipids derived from plasma membranes of control (white columns) and *ras*-transformed (black columns) fibroblasts. Values are means ± SD from three determinations in three separate experiments. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PG, phosphatidylglycerol; SM, sphingomyelin.

control and *ras*-transformed cells were manipulated. One series of acceptor vesicles was prepared by increasing by about 70% the native content of PE in the vesicles containing lipids from the two cell lines. The aim of these experiments was to make the level of PE in the model membranes almost as high as PC but still to let PC remain the major membrane fraction. The phospholipid composition of the manipulated vesicles is shown in Fig. 2. It is evident that the percentage of the other phospholipids was decreased in the manipulated model membranes due to the significant augmentation of PE, which became a major lipid fraction (Fig. 2). Another series of vesicles were prepared in a similar way, only in this case the content of SM was augmented (Fig. 3).

Elevation of PE in acceptor vesicles was accompanied by higher transfer of labeled PC to the manipulated acceptor membranes compared to non-manipulated ones (Table 1). In contrast, elevation of SM in acceptor membranes induced a reduction of NBD-PC transfer (Table 1).

In parallel experiments, structural organization of membrane lipids in all series of acceptor vesicles was investigated using DPH as a fluorescent probe (Table 2). Preliminary studies showed that the presence of 1 mol% of NBD-lipid analogues in model membranes did not affect lipid structural organization. The results showed that the S_{DPH} was reduced in all series of acceptor vesicles enriched with PE, and was elevated in all vesicles enriched with SM compared to non-manipulated vesicles (Table 2). Comparing the values of S_{DPH} with the alterations of PC transfer, we observed that the transfer was higher towards model membranes with reduced S_{DPH}. Also, a decreased transfer was measured in acceptor membranes where the

Table 2. S_{DPH} of acceptor vesicles containing lipids from plasma membranes of control and *ras*-transformed fibroblasts

Manipulation of acceptor vesicles	Control cells	*ras* cells
None	0.7212 ± 0.0018	0.6374 ± 0.0015
PE	0.6987 ± 0.0021*	0.6212 ± 0.0017*
SM	0.7448 ± 0.0027*	0.6542 ± 0.0033*
Phospholipase D	0.6803 ± 0.0031*	0.6123 ± 0.0025*

Control vesicles contain lipids derived from control cells. *ras*-vesicles contain lipids from *ras*-transformed cells. Values are means ± SD from three separate experiments performed in triplicates; * $p < 0.001$.

value of S_{DPH} was increased compared to non-manipulated membranes (Tables 1 and 2).

Further studies were aimed at modulation of the level of PA – a membrane component of significant physiological importance (English et al. 1996), which has been reported to be elevated in *ras*-transformed cells (Lacal and Carnero 1994). The level of PA was increased in acceptor membranes by treatment with exogenous phospholipase D, which modifies phospholipid headgroups and induces accumulation of PA. As a result of phospholipase D treatment, the fraction of PA was augmented from traces to 13.36% in vesicles containing control cell lipids, and to 18.79% in *ras* cells vesicles. These lipid modifications were accompanied by an increase of lipid transfer (Table 1) and a decrease of S_{DPH} (Table 2) in each series of acceptor vesicles.

Discussion

Most studies concerning interactions of lipid vesicles with cells have focused largely on the transfer of vesicle components to cell membranes via the mechanism of fusion or endocytosis (Hoekstra and Wilschut 1988; Tullius et al. 1989). By definition, these processes imply cellular uptake of both vesicle lipids and the aqueous contents. However, it is also clear that certain amounts of lipid can be transferred between cells and vesicles without uptake of the internal cellular or vesicular contents (Struck and Pagano 1980). Although this mechanism is not clearly understood, there is abundant evidence that, under certain conditions, a significant exchange of vesicle and cellular phospholipid can also take place (Zachowski and Gaudry-Talarmain 1990; Savill 1996). There are different presumptions on the mechanism of lipid exchange (Nichols and Pagano 1981; Bratton et al. 1997; Pradhan et al. 1997). The aim of this study was to shed some light on the mechanism of spontaneous lipid transfer between membranes and, more specifically, on the role of phospholipid composition and structural organization of the membranes participating in this process. For this purpose, model membranes were used containing

plasma membrane phospholipids isolated from control and *ras*-transformed cells, which differed in their phospholipid composition (Momchilova et al. 1998). Our experimental model, which contained only lipids derived from cellular plasma membranes, offered a good possibility to differentiate between the role of membrane lipids and other plasma membrane-components, such as proteins. In order to analyze whether the observed differences in NBD-PC transfer could be due to the different lipid composition of the two types of acceptor membranes, we manipulated the level of the lipid fractions showing the most pronounced differences between control and *ras* cells – PE and SM. The eventual effects of the minor fractions of phosphatidylserine, phosphatidylinositol, and phosphatidylglycerol were not analyzed because the differences in their level between control and *ras* cells were in the range of 3–5 %. It is not likely that such differences in the levels of minor fractions could affect the lipid exchange processes between membranes. The results showed that spontaneous lipid transfer was higher to non-manipulated acceptor vesicles which contained higher levels of PE and SM and were more fluid. These were the model membranes prepared of lipids from plasma membranes of *ras* cells. In present studies we also analyzed whether the differences in lipid composition were accompanied by the corresponding alterations in the membrane structural order. In a previous paper (Momchilova and Markovska 1999) we reported that almost all monounsaturated and polyunsaturated fatty acids were elevated in PE of *ras* cells compared to control cells. Thus, the fatty acid composition of PE elevated in the transformed cells, could be a possible reason for the lower structural order of model membranes containing lipids from *ras* cells. The effect of PE on membrane order could as well be due to the fact that PE in general perturbs the bilayer organization of lipid membranes because of its non-bilayer behavior (Huang et al. 1999). It is quite likely that the observed fluidization of acceptor membranes might occur as a factor facilitating the process of spontaneous lipid transfer. This hypothesis was tested by augmentation of SM in acceptor membranes because this lipid is known to reduce membrane fluidity (Ahmed et al. 1997). The results confirmed this view, because in both types of acceptor vesicles with increased SM a reduction of PC transfer compared to the non-manipulated vesicles was observed (Table 1). Preliminary investigations in our laboratory indicated that an increase of cholesterol in acceptor model membranes reduced the transfer of labeled lipids to such vesicles (unpublished data). All these observations imply that structural organization of the membranes participating in spontaneous lipid inter-membrane exchange is an important factor influencing the rate of the process.

To analyze in more detail how membrane composition and structural order affect spontaneous phospholipid transfer, model membranes prepared of phospholipids from either control or *ras*-transformed fibroblasts were treated with exogenous phospholipase D. This treatment induced a reduction mainly of PC, accompanied by an increase of PA. PA is a minor membrane component and a potent participant in the *ras* signal pathway (Lacal and Carnero 1994). The PA molecules are known to affect local membrane curvature, thus facilitating the processes of fusion and fission and perturbing membrane organization (Kooijman et al. 2005).

Possibly, this bilayer-perturbing behavior of PA underlies the observed effect of fluidization of phospholipase D-treated membranes. These observations confirmed the presumption that the disturbances of membrane structural organization affect spontaneous lipid transfer between membranes.

Taken together, the results obtained in these studies showed that spontaneous phospholipid transfer to model membranes was affected by changes in membrane lipid composition and structural organization. The lipid transfer was higher to *ras* cells model membranes containing a higher level of PE, and to all acceptor vesicles with elevated content of PE, suggesting that the presence of PE may also play a role in spontaneous lipid exchange between membranes. In addition, we reported in our previous studies that PE was not only elevated in membranes of *ras* fibroblasts, but was also present in higher quantity in the outer plasma membrane monolayer due to activated scramblase and partially inactivated aminophospholipid translocase in *ras* cells (Momchilova et al. 2000). This specific localization of PE in plasma membranes of *ras* cells may have an important physiological significance, which could as well be related to lipid uptake. It is also possible that the higher capacity of *ras* cells membranes to uptake exogenous lipid molecules is a compensatory mechanism by which the cells partially compensate the loss of plasma membrane lipids due to stimulated endocytosis (Lacal and Carnero 1994).

Of interest is also the fact that phospholipid exchange between vesicles and cells could be related to a similar phenomenon, which occurs between serum lipoproteins and cells (Sleight and Pagano 1985). Although the physiological importance of spontaneous phospholipid transfer between cells is not entirely understood, the model membrane system containing cell-derived lipids certainly provides a useful tool for investigation of the processes of lipid exchange.

In conclusion, the results presented in this paper indicate that spontaneous lipid transfer between membrane bilayers is a complex process which depends on the composition and structural organization of acceptor membranes. We presume that membrane fluidization and probably elevated content of PE facilitate spontaneous transfer of phospholipids between membranes. Such studies may be useful for a better understanding of the complex cell-to-cell interactions and spontaneous exchange of phospholipids between cellular membranes.

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