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Incorporation of exogenous phosphatidylcholine in the plasma membrane of MDCK cells by a specific transfer protein

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The possibility to introduce exogenous phosphatidylcholine (PC) in the plasma membrane of Madin-Darby canine kidney (MDCK) cells other than by fusion of liposomes with virus-infected cells (Van Meer, G. and Simons, K. (1983) J. Cell Biol. 97, 1365–1374) was studied. Monolayers of confluent MDCK cells grown on a permeable support were exposed to unilamellar vesicles of dipalmitoylphosphatidylcholine (DPPC), a phospholipid that does not exchange spontaneously, and were incubated with or without the PC-specific transfer protein (PC-TP), at 4 and 37°C. Added either on the apical or basolateral side of monolayers grown in the presence of [^{14}C]choline, PC-TP stimulated the transfer of ^{14}C-labeled PC from the cell membrane to the liposomes, even at 4°C. Conversely, PC-TP promoted the transfer, by a temperature-dependent process, of [^{3}H]DPPC from liposomes to the cell plasma membrane. The amount of DPPC imported at 37°C was higher than 100 pmol/well for apical incubations. The data demonstrate that, in MDCK cells: (a) PC-TP can modify the PC species present in the plasma membrane; (b) PC accounts for a significant amount of the polar lipids present in the external leaflet of the apical membrane domain.

Introduction

Besides a polarized distribution of transport proteins, enzymes and receptors, the apical and basolateral domains of the plasma membrane of renal epithelial cells differ in their lipid composition and physical state [1–3]. This lipid asymmetry depends on the intactness of the tight junctions [4,5] which defines an apical domain whose external leaflet is enriched in sphingolipids [2,3,6]. Recent evidences indicate that the composition and/or physical state of the apical external leaflet may control the activity of Na-coupled transport processes [7–10]. In particular, it has been suggested that the phosphatidylcholine (PC) content and the nature of the acyl chains of the PC species present in brush border membranes regulate the Na/phosphate co-transport activity [11,12].

Confluent monolayers of cultured Madin-Darby canine kidney (MDCK) cells retain many of the differentiated properties associated with the kidney tubular epithelium [13,14]. Their biochemical polarity is well established for membrane proteins [15,16], and viruses budding from either apical or basolateral plasma membrane domains have different phospholipid compositions [17], in accordance with a marked asymmetry in the membrane physical state [18]. They possess a Na/phosphate co-transport system whose activity is affected by changes in membrane fluidity [19]. Up to now, introduction of exogenous PC in the plasma membrane of MDCK cells has been achieved via fusion of liposomes with the plasma membrane of cells infected by influenza viruses [20,21], i.e, a condition that precluded any physiological study. The following study was performed to determine if, as in non polarized cells [22–24], the PC-specific transfer protein (PC-TP) could be used to introduce exogenous PC in the plasma membrane domains of MDCK cells grown as a monolayer on permeable support. Because liposomes adsorb at the membrane surface of these cells, the experiments were done using dipalmitoylphosphatidylcholine (DPPC), a PC species for which there is, if any, very little PC transfer to the cell membrane [21]. It was

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found PC-TP mediated transfer of DPPC to the apical membrane of MDCK cells is as efficient as membrane fusion in infected cells to introduce exogenous PC. This provides an usefull tool for studies on co-transport/membrane PC relationships in renal epithelial cells.

Experimental procedures

Materials

[*Me*-^{14}C]Choline chloride (82 Ci/mmol), glycerol tri[1-^{14}C]oleate (60 Ci/mol), 1,2-di[1-^{14}C]palmitoylphosphatidylcholine (110 Ci/mol), 1-palmitoyl-2-[1-^{14}C]oleoylphosphatidylcholine (52 Ci/mol) were obtained from Amersham. 1,2-Dipalmitoylphosphatidyl[*N*-*Me*-^{3}H]choline and $K_2H^{32}PO_4$ (900 mCi/mmol) were purchased from New England Nuclear. The PC-specific transfer protein (PC-TP) was Isolated from beef liver and stored as described earlier [23]. Phosphatidic acid (PA) from egg yolk, 1,2-dipalmitoylphosphatidylcholine, and 1-palmitoyl-2-oleoylphosphatidylcholine were obtained from Sigma. Plasticware and tissue culture-treated 3 μm pore polycarbonate membranes (24 mm Transwell) were from Costar (Cambridge, MA, U.S.A.). All other biochemicals used were of the highest purity available and obtained from regular commercial sources.

Cell cultures

MDCK cells were obtained from Flow Labs (Irvine, U.K.) at passage 65. They were used between passages 78 and 80. Cells were first grown to confluence on plastic culture dishes, in a 5% CO_2/95% air atmosphere in a 50:50 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 medium supplemented with 10% fetal calf serum, at 37°C [18,19]. After two passages, cells were seeded on 3 μm pore Costar Transwell and grown for 4 days with the complete medium in the upper and lower chambers. The medium was then changed for fresh medium containing (lower chamber) or not (upper chamber) fetal calf serum. The medium was changed in a similar way 24 h prior to the experiment (between days 10 and 12 after seeding). Confluence of cultures at this stage was previously established (Giocondi and Le Grimellec, submitted). For ^{32}P labeling of lipids the cells were incubated for 3 days with apical and basolateral medium containing 5 μCi/ml ^{32}P. Labeling of choline-containing phospholipids was obtained by adding 1 μCi/ml [^{14}C]choline for 48 h to the lower chamber medium.

Unilamellar vesicles

Small unilamellar vesicles composed of 90% DPPC (or in few experiments POPC) and 10% PA were obtained by the ethanol injection technique [25,26]. Briefly, 0.45 mg dried phospholipids were solubilized in 20 μl ethanol (29 mmol) and the solution was injected in 125 mM NaCl, 5 mM KCl, 20 mM Na_2HPO_4, 5 mM NaH_2PO_4, 0.5 mM $CaCl_2$, pH 7.4 buffer (PBS Ca-buffer) heated at 55°C under stirring. The resulting liposome suspension was dialysed overnight, at 4°C against 800 ml of the same buffer. When used, 0.1 μCi [^{3}H]DPPC and 12.5 nCi of glycerol tri[1-^{14}C]oleate were added before the solubilization in ethanol.

Conditions of PC transfer

The experiments were carried out at either 4 or 37°C. When using [^{14}C]choline-labeled cells, the monolayers were washed three times with ice-cold PBS-Ca buffer. Liposome suspension (14 nmol PC/ml) pre-equilibrated at the chosen temperature and containing, or not, the PC-TP (1.4 μmol) was added, at zero time, either in the apical (1.5 ml) or in the lower (2.6 ml) chambers. Prior to the experiments, the PC-TP/liposome suspension was incubated, with gentle stirring, for 2 h at 37°C. The same procedure was followed for control liposomes. The chamber not receiving the liposome suspension was filled with PBS-Ca buffer. Transwell chambers disposed in 6 well cluster plates, were placed in a Lab Line (Lab Line Inc., IL) incubator, equipped with an orbital shaker set at 50 rpm. At $t = 1$, 15 and 30 min 400 μl of the liposome suspension were collected and extracted according to Bligh and Dyer [27]. When incubating with [^{3}H]DPPC liposomes, the filters, after the 30 min incubation period, were washed three times with ice-cold buffer, their bottom wipped off with tissue paper, and were disposed on an aluminium block maintained at 0°C. The cells were scraped off with a razor blade and the lipid extracted.

Lipid analysis

Lipids were extracted using the method of Bligh and Dyer [27] slightly modified, as previously described [3,7]. Just before extraction, known amounts of either [^{3}H]- or [^{14}C]DPPC, according to the tracer used during the experiment, were added to be used as internal standards. Extracts were evaporated to dryness under nitrogen and solubilized in chloroform/methanol mixture (2:1, v/v). Thin-layer chromatography was done on precoated silica gel thin-layer plates (Whatman LK5) impregnated with boric acid using chloroform/ethanol/water/triethylamine (30:35:6:35, v/v) [28]. Individual components were detected under UV light after spraying with a solution of 5 mg of primulin in 100 ml of acetone/water (4:1, v/v) and identified by comparison with authentic standards. The spots were scraped off and counted. The phosphorus content of unlabeled cells phospholipids was determined according to Mrsny et al. [29].

Results

PC-TP stimulates the transfer of PC between cell membrane and liposomes

Growing the MDCK cell monolayers in the presence of [^{32}P]monophosphate revealed that PC represented, under the conditions used, nearly half of the cell phospholipid: values for PC, phosphatidylethanolamine, phosphatidylinositol, sphingomyelin, phosphatidylserine and lysoPC were 48, 24.6, 10.5, 5.5, 3.8 and 1.0% ($n = 4$), respectively. A similar value was obtained from chemical determination of phosphate (134 nM P_i/well for total phospholipids vs. 65 nM P_i in PC). Using [^{14}C]choline to label choline-containing species, most of the label (81.0 ± 0.3%, $n = 3$) was, in accordance with literature [30], recovered from the lipophilic phase of the cell extract. Phosphatidylcholine accounted for 85% of the labeled lipid species, whereas lyso-PC represented less than 1.5% of the total. Incubation of the monolayers at 4 °C prevents endocytotic and exocytotic processes [31]. As shown in Fig. 1, the addition of PC-TP to the apical (A) or the basolateral (B) medium in the presence of liposomes made of DPPC enhanced the exit of labeled compound from [^{14}C]choline-labeled monolayers, at 4°C. These data suggest that PC-TP had promoted an exchange of PC between the MDCK cell membrane and the liposomes. In accordance with that view, replacement of DPPC by POPC for which efficiency of transfer by PC-TP must be increased [32], resulted in a marked stimulation of the ^{14}C release (Fig. 1).

As illustrated by Fig. 2, extraction of the DPPC liposome suspensions followed by TLC confirmed that PC-TP stimulated the transfer of ^{14}C-labeled PC from the cell membrane to the liposomes. This transfer was time dependent and was markedly enhanced by raising the temperature of incubation to 37°C: PC-TP-induced

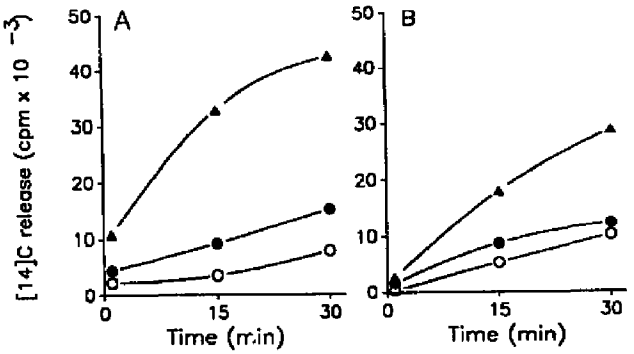

Fig. 1. Effect of addition of PC-TP on the release of ^{14}C by MDCK cells pre-incubated in [^{14}C]choline. Monolayers were incubated at 4°C with liposomes suspended in PBS-Ca in the presence (closed symbols) or not (open symbols) of PC-TP (see Experimental procedures). Aliquotes of the liposome suspensions were collected at 1, 15 and 30 min and counted. (A) Apical incubation; (B) basolateral incubation. Circles, DPPC liposomes; Triangles, POPC liposomes.

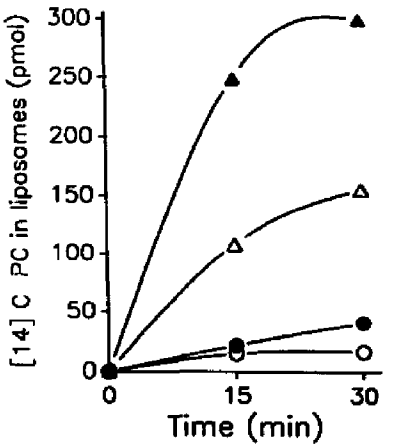

Fig. 2. PC-TP transfers [^{14}C]PC from cells to liposomes. Cells were incubated, on their basolateral side, with (closed symbols) or without (open symbols) PC-TP containing DPPC liposome suspensions at either 4 °C (circles) or 37°C (triangles). Aliquotes of the liposome suspensions were collected, extracted by the Bligh and Dyer method, and the radioactivity in the PC species was determined after a separation by TLC.

exit of PC was 24 and 145 pmol at 4 and 37°C, respectively, for 30 min incubations.

PC mediated transfer from liposomes to the cell membrane

To examine this transfer, MDCK cell monolayers were incubated for 30 min with [^{3}H]DPPC liposomes and the radioactivity contained in the hydrophobic part of the cell extract was determined. As shown by Fig. 3, PC-TP increased the radioactivity of the cell extracts when added either on the apical or the basolateral side. These data also showed that in absence of PC-TP, the radioactivity found when incubating on the apical side, exceeded that determined from basolateral incu-

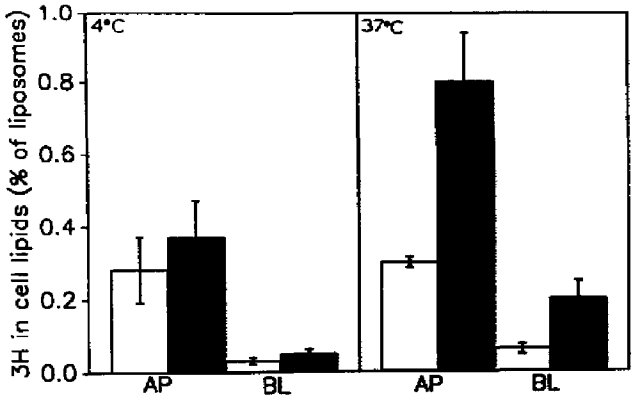

Fig. 3. PC-TP transfers [^{3}H]DPPC from liposomes to cell monolayers. Following 30 min incubation at 4 °C (left) or 37°C (right) with (full bars) or without (open bars) PC-TP either on the apical or the basolateral side of the monolayer, cellular lipids were extracted and the corresponding radioactivity determined. The data are presented as percentage of the total radioactivity contained in the liposome suspension. Values are means ± standard error of three experiments.

TABLE I

PC-TP stimulated transfer of DPPC from liposomes to MDCK cell monolayers

Confluent monolayers were incubated with 14 nmol/ml of DPPC liposomes labeled with [^{3}H]DPPC in the presence (+PC-TP) or the absence (−PC-TP) of the transfer protein (1.4 nmol/ml). The amount of PC transferred was calculated from the specific activity of DPPC in liposomes. Values are expressed as means ± standard error of three experiments.

Incubation	nmol PC transferred/well	
	apical	basolateral
(A) 4 °C		
−PC-TP	0.09 ± 0.01	0.03 ± 0.01
+PC-TP	0.15 ± 0.04	0.03 ± 0.01
(B) 37 °C		
−PC-TP	0.13 ± 0.02	0.05 ± 0.01
+PC-TP	0.27 ± 0.05	0.09 ± 0.01

bations and was poorly affected by the temperature. Determination, from the radioactivity found in PC after TLC, of the absolute amount of DPPC originating from the liposomes and present in the cell extracts confirmed, with the exception of the basolateral data at 4°C, the stimulation of the transfer of DPPC to the cell membranes by PC-TP (Table I). In that series of experiments, the maximal effect, corresponding to a transfer of 140 pmol DPPC in 30 min (difference between incubations with and without PC-TP), was observed for apical incubations at 37°C.

Further support to the PC-TP stimulated transfer of DPPC was gained from experiments in which glycerol tri[^{14}C]oleate in tracer dose was added, as a second non-exchangeable marker [33], to [^{3}H]DPPC-labeled liposomes. As shown in Table II, the ^{3}H/^{14}C ratio in cell extracts was significantly enhanced by the presence

TABLE II

^{3}H/^{14}C ratio in lipid extracts of MDCK cell monolayers incubated with [^{3}H]DPPC liposomes containing glycerol tri[1-^{14}C]oleate

The corresponding ratio for liposomes before their addition to the cell monolayer was 8.0 ± 0.4. MDCK cell monolayers were incubated for 30 min with DPPC liposomes containing glycerol trioleate in trace amount in the presence or absence of PC-TP. At the end of the incubation cells were washed and their lipid extracted (see Materials and Methods). Aliquots of the lipid extracts were collected and counted. Values are expressed as means ± standard error ($n = 3$).

Incubation	^{3}H/^{14}C ratio	
	apical	basolateral
(A) 4 °C		
−PC-TP	7.3 ± 0.3	6.9 ± 0.7
+PC-TP	8.6 ± 0.6	12.4 ± 0.3
(B) 37 °C		
−PC-TP	9.2 ± 1.5	6.5 ± 0.8
+PC-TP	12.8 ± 1.2	15.5 ± 1.3

of PC-TP in the incubation medium. Raising the temperature from 4 to 37°C increased the value taken by this ratio for both the apical and basolateral incubations. It was, however, noticeable that (a) except for the apical incubation at 37°C, the values obtained in the absence of PC-TP were lower than those of the liposomes before their addition to the cell monolayers; (b) the higher ^{3}H/^{14}C ratios were obtained from basolateral incubations.

Discussion

The present experiments demonstrate that PC-TP can exchange PC between liposomes and the apical or, altough to a lesser extent, the basolateral domains of the plasma membrane of MDCK cells grown as a confluent monolayer on a permeable support.

This demonstration was based on the observations that PC-TP Increased (a) the exit of PC from [^{14}C]choline-labeled cells; (b) the amount of [^{3}H]DPPC associated to the cells during incubation with labeled liposomes; (c) the ^{3}H/^{14}C ratio of cells incubated with [^{3}H]DPPC/ glycerol tri[^{14}C]oleate liposomes. In order to establish unambiguously the phenomenon, we used liposomes made of DPPC for the exchange and short (30 min) incubation periods in order to limit both endocytosis and PC metabolism at 37°C [34].

In all the experiments, the radioactivity associated with cells incubated with liposomes but without PC-TP on the apical side exceeded that found for basolateral incubations. In accordance with the view that this radioactivity essentially represented liposome adsorption at the cell surface, the corresponding amount of phospholipid, i.e., 90 pmol/well, was not significantly modified by raising the temperature from 4 to 37°C and was in the range of the values reported for liposome adsorption on MDCK cell monolayers [5,21]. Two factors can account for the lower adsorption on the basolateral side. First, the fact that the bottom of the chambers were wiped off with tissue paper after the incubations (see Materials and Methods). Secondly, one has to remind that the area of pores in 3 μm polycarbonate membranes represent only 14% of the total area [35].

Even at 4°C, a temperature at which endocytic processes are blocked, PC-TP, when added in the apical medium, stimulated the exit of [^{14}C]labeled PC from the cells and the transfer of [^{3}H]DPPC from liposomes to the cells. The transfer protein furthermore increased the ^{3}H/^{14}C ratio of cells incubated with DPPC/glycerol trioleate liposomes. The absolute amount of PC transferred at 4°C corresponded to about 40% of the transfer obtained at 37°C. Although the exit of cell PC, the ^{3}H in the total lipid extract and the DPPC/glycerol trioleate were increased by incubation at 4°C with PC-TP on the basolateral side, we

were not able to observe a significant increase in ^{3}H content of PC species after TLC of cell extracts. This was likely due to the low signal originating from these samples. Two remarks have to be made concerning the values taken by the [^{3}H]DPPC/glycerol tri[^{14}C]oleate ratio. First, we repeatidly observed that, even at 4°C, the value determined from cell extracts in absence of PC-TP was lower, especially on the basolateral side, than the value of the liposome suspension. This might indicate that a part of the glycerol trioleate has been hydrolyzed at the cell surface, leading to an entry of oleic acid into cells. The presence of a triacylglycerol lipase in the plasma membrane of MDCK cells was previously suggested to explain the rapid hydrolysis of glycerol trioleate after liposome fusion in cells infected with Influenza virus [21]. It is possible that, in MDCK cells grown on a permeable support, the triacylglycerol lipase was predominantly localized in the basolateral membrane domain. The second point is that the $^{3}H/^{14}C$ ratios approached higher values for basolateral incubations whereas the PC-TP stimulated exchange was lower on this side. It is likely that this resulted from the much lower non specific binding of the liposomes on that side of the monolayer.

Raising the temperature of incubation to 37°C, resulted in a PC-TP dependent transfer of PC from liposomes to cells of approx. 140 and 40 pmol DPPC/well in 30 min for apical and basolateral incubations, respectively. Although of the same order, this last value remained lower than that obtained in the series where cells to liposomes transfer was examined. Variability between batchs of cultures as well as inevitable material losses when scraping cells from the filters were likely explanations for the observed difference. The extent of PC transfer was similar when using three times less (4 nmol/ml) DPPC in the incubation medium (not shown), indicating that the transfer was not limited by the amount of exogenous PC. With respect to the apical membrane, the efficiency of the transfer compares well with the amount of exogenous PC that can be introduced in the plasma membrane by fusion of liposomes with viruses-infected cells [21]. For MDCK cells grown on Millipore 20 mm diameter filters it was estimated that the apical outer leaflet contains maximally 1.2–1.9 nmol of polar lipids [6]. This would correspond to 1.8–2.8 nmol for Transwell filters, considering that their distribution in cells grown on both supports is similar [36]. Using NBD-PC it was previously shown that the flip-flop rate of PC is low in MDCK or A 6 epithelial cells [4]. Therefore, our data strongly suggest that the outer leaflet contains at least 5 to 8% of PC. This makes the apical membrane of MDCK cells to resemble that of intestinal cells which is rich in glycolipids but also contains significant amounts of PC on its external leaflet [37]. The liposome to cell transfer of DPPC at the basolateral side was 3–4-times lower than at the apical side, despite the fact that basolateral membranes must contain a larger amount of PC on their external leaflet. However, it should be realised that, as mentioned above, the area available for the exchange at the basolateral side is less than 7-times the area of the filter, i.e., approx. 10-times lower than that of the apical side [6]. Additionally, the pore configuration is likely to result in an unstirred layer [38], thus limiting the efficiency of the exchange.

In conclusion, these data show that PC-TP, as in non-polarized cells, allows to replace significant amounts of the PC of the plasma membrane of MDCK cells by exogenous PC. They provide a basis for studies on relationships between cell physiology and membrane PC in renal epithelial cells.

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