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Natural Phosphatidylcholine Is Actively Translocated across the Plasma Membrane to the Surface of Mammalian Cells*

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The cell surface of eukaryotic cells is enriched in choline phospholipids, whereas the aminophospholipids are concentrated at the cytosolic side of the plasma membrane by the activity of one or more P-type ATPases. Lipid translocation has been investigated mostly by using short chain lipid analogs because assays for endogenous lipids are inherently complicated. In the present paper, we optimized two independent assays for the translocation of natural phosphatidylcholine (PC) to the cell surface based on the hydrolysis of outer leaflet phosphoglycerolipids by exogenous phospholipase A_2 and the exchange of outer leaflet PC by a transfer protein. We report that PC reached the cell surface in the absence of vesicular traffic by a pathway that involved translocation across the plasma membrane. In erythrocytes, PC that was labeled at the inside of the plasma membrane was translocated to the cell surface with a half-time of 30 min. This translocation was probably mediated by an ATPase, because it required ATP and was vanadate-sensitive. The inhibition of PC translocation by glibenclamide, an inhibitor of various ATP binding cassette transporters, and its reduction in erythrocytes from both Abcb1a/1b and Abcb4 knockout mice, suggest the involvement of ATP binding cassette transporters in natural PC cell surface translocation. The relative importance of the outward translocation of PC as compared with the well characterized fast inward translocation of phosphatidylserine for the overall asymmetric phospholipid organization in plasma membranes remains to be established.

The distribution of lipids across the eukaryotic plasma membrane bilayer is asymmetric with the choline phospholipids sphingomyelin and phosphatidylcholine (PC)[1] at the cell surface and the aminophospholipids phosphatidylserine (PS) and phosphatidylethanolamine (PE) at the inside (1, 2). PS and PE are continuously translocated from the exoplasmic to the cytoplasmic leaflet of cellular membranes by proteins belonging to a subfamily of P-type ATPases (3, 4). Inward translocation of PS is essential to prevent PS cell surface signaling, which induces blood coagulation and serves as a signal for cell-cell recognition, *e.g.* the removal of apoptotic cells by macrophages. An additional function of lipid translocation has emerged recently. Evidence from mammalian and yeast cells suggests that ATP-dependent inward translocation of phospholipids by the aminophospholipid translocase affects membrane curvature and is a, or the, driving force for the formation of endocytic vesicles at the plasma membrane (4–6). The fact that yeast expresses five P-type ATPase family members in different compartments of the exocytic and endocytic transport pathways suggests the possibility that all membrane budding from sphingolipid- and cholesterol-rich membranes depends on the mass translocation of membrane phospholipids.

The bulk membrane phospholipid in mammalian cells is PC, which constitutes 25–50 mol % of the membrane lipids. However, it is unclear if cells possess mechanisms for the plasma membrane translocation of PC as for PS and PE. PC is a cylindrical lipid with a low tendency to flip across membranes, with a half-time of days in model membranes. Indeed, using natural PC (7–9) or spin-labeled and fluorescent (C_6-NBD-) short chain PCs (10, 11), typical half-times for inward translocation have been reported of hours (as compared with minutes for PS) in the erythrocyte membrane as a model plasma membrane. Still, in some cells the short chain PCs displayed rapid inward translocation (12) in an ATP-dependent and *N*-ethylmaleimide-sensitive manner (13), as they do in yeast (4, 14). Either some cells possess a specific inward PC translocator or some aminophospholipid translocases are not strictly specific for PS and PE. In erythrocytes, aminophospholipids also translocate in the opposite, outward direction but much slower than inward: short chain PS and PE displayed ATP-dependent inward and outward transport with half-times of 3 and 35 min, respectively, for the inward direction and 58 and 77 min for the outward direction (15, 16). Similarly, the outward movement of C_6-NBD-PC was ATP-dependent (15). First hints for the identity of one PC outward translocator came from the finding that PC secretion into mouse bile depended on the presence of the ATP-binding cassette (ABC) transporter Abcb4 (17), and that this liver transporter enhanced transport of newly synthesized PC to the surface of transgenic fibroblasts (18). In studies on short chain lipids, human ABCB4 was found to be specific for PC, whereas, unexpectedly, the closely related multidrug transporter ABCB1 translocated a wide variety of short chain lipids (19, 20), including the short chain PC platelet activating factor (21, 22). However, in erythrocytes the outward translocation of C_6-NBD-PC and -PS was found to be mediated by an alternative ABC transporter, ABCC1 (23). Because the expression of ABCB4 is rather specific for liver and there is no

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[1] The abbreviations used are: PC, phosphatidylcholine; C_6-NBD-, *N*-6-(7-nitro-2,1,3-benzoxadiazol-4-yl)-aminohexanoyl-; PS, phosphatidylserine; PE, phosphatidylethanolamine; SUV, small unilamellar vesicle; PLA_2, phospholipase A_2; PCTP, phosphatidylcholine transfer protein (nomenclature of ABC transporters: ABCB1 = MDR1, ABCB4 = MDR3, ABCC1 = MRP1, ABCC7 = CFTR, ABCG2 = BCRP); DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; BSA, bovine serum albumin; AMP-PNP, adenosine 5′-(β,γ-imino)triphosphate; BFA, brefeldin A.

convincing evidence that ABCB1 and ABCC1 translocate natural, long chain lipids, the question remains whether natural PC is actively translocated across the plasma membrane of non-hepatocytes.

Outward translocation of natural PC has been reported in rat and human erythrocytes (24–26). The process appeared restricted to newly synthesized PC, sensitive to the arginine modifying reagent phenylglyoxal, and insensitive to vanadate (26), suggesting protein-mediated but energy-independent PC translocation as it was also proposed for bile canalicular membranes (27). In the present study we re-evaluated the translocation of natural PC from the inside to the outside of the plasma membrane. For this, we optimized two independent assays for measuring the fraction of intracellularly labeled PC that arrived at the cell surface, the hydrolysis of cell surface PC with phospholipase A_2 (1) and exchange of outer leaflet PC against liposomal PC by the PC transfer protein (28). We investigated PC cell surface translocation in erythrocytes and in fibroblasts and present evidence that PC is actively translocated across mammalian plasma membranes with characteristics that would be in accordance with an involvement of ABC transporters.

EXPERIMENTAL PROCEDURES

Materials—The radioactive fatty acids [1-^{14}C]arachidonic acid (50 Ci/mol), ([1-^{14}C]- and [U-^{14}C]palmitic acid (50 Ci/mol and 500 Ci/mol, respectively), and [1-^{14}C]oleic acid (50 Ci/mol) were from Amersham Biosciences; L-[*palmitoyl*-1-^{14}C]carnitine chloride (50 Ci/mol) was from PerkinElmer Life Sciences, [^{32}P]H_3PO_4 in H_2O was from ICN (Zoetermeer, The Netherlands), NBD-labeled lipids were purchased from Avanti Polar Lipids (Alabaster, AL). Chemicals and enzymes, if not indicated otherwise, were from Sigma and used in the highest purity available. Indomethacin was from ICN (Aurora, OH). PSC833 was a kind gift from Novartis Pharma AG (Basel, Switzerland). Ko143 was a kind gift from Alfred Schinkel (NKI, Amsterdam, The Netherlands). Silica TLC plates were from Merck (Darmstadt, Germany), organic solvents were from Riedel de Haën (Darmstadt, Germany), and cell culture media were from Invitrogen.

Lipid Analysis—Lipids from intact cells were extracted according to Bligh and Dyer (29), dried under nitrogen, and separated by two-dimensional thin layer chromatography with the first dimension in chloroform, methanol, 25% ammonia (65:25:4), followed by chloroform: methanol:acetone:acetic acid:water (50:20:10:10:5) (30) (Fig. 1). [^{32}P]Phosphate-labeled lipids were separated before TLC on Accell Plus CM anion exchange SepPak columns (Waters, Etten-Leur, The Netherlands). Briefly, columns were equilibrated with $CHCl_3$:MeOH (2:1), the lipids were loaded in a few drops of $CHCl_3$:MeOH (2:1), and the uncharged lipids were eluted with 4 ml of 1 mM ammonium acetate in $CHCl_3$:MeOH:H_2O (3:6:1). 1.2 ml of 4 mM HCl was added and a phase separation was performed. Anionic lipids were eluted with increasing ammonium acetate concentrations. TLC plates were exposed to a phosphorimager screen (BAS-MS or BAS-TR, Fuji Medical Systems, Stanford, CT) for 2 days and scanned with a Personal Molecular Imager FX System (Bio-Rad). The fluorescence of C_6-NBD-PC-labeled cells was measured with a STORM imaging system (Amersham Biosciences). Quantifications were performed with Quantity One Software (Bio-Rad). Total phospholipids were quantified by phosphate determination after scraping the iodine-stained lipid spots from two-dimensional TLC plates (31).

Cells and Animals—Wt1.2 mouse fibroblasts (32) were cultured in Dulbecco's modified Eagle's medium containing Glutamax-I, 4.5 g/liter glucose, and 10% heat-inactivated fetal calf serum under 5% CO_2. Human erythrocytes were obtained from healthy volunteers by venipuncture, from anesthetized mice by heart puncture or from the eye background with sodium heparin as anticoagulant. Animal experiments were performed according to the guidelines of the Dutch government concerning animal care. Erythrocytes were collected by centrifugation and the buffy coat was removed by five washes with buffer A (140 mM NaCl, 5 mM KCl, 10 mM Hepes, pH 7.4). Cells were resuspended in buffer A, 15 mM glucose, and used within 2 days. Mouse blood was investigated on the day of blood withdrawal. Blood from the double Abcb1a/1b$^{-/-}$ and triple Abcb1a/1b$^{-/-}$, Abcc1$^{-/-}$ (33, 34), Abcb4$^{-/-}$ (17), Abcc7$^{-/-}$ (35), and Abca1$^{-/-}$ (36) knockout mice was generously provided by Alfred Schinkel (NKI, Amsterdam), Ronald Oude Elferink (AMC, Amsterdam), Hugo de Jonge (EUR, Rotterdam), and Folkert Kuipers (RUG, Groningen), respectively. In each experiment, performed in duplicate, three knockout animals were compared with three matched control animals.

Energy-depletion of Human Erythrocytes—Human erythrocytes were incubated for 2 h in buffer A containing 50 mM deoxyglucose and 5 mM KF (37) before a 2-h incubation in the presence of the label, [^{14}C]palmitoylcarnitine (38) or [^{14}C]arachidonic acid, where we used 0.2 μCi in energy-depleted and 0.02 μCi of [^{14}C]arachidonic acid per 100 nmol of total phospholipid in fresh erythrocytes.

Preparation of Resealed Erythrocyte Ghosts—Ghosts were prepared from fresh erythrocytes by hypotonic shock. One volume of the erythrocyte pellet was diluted into 4 volumes of ice-cold lysis buffer (9 mM KCl, 4.5 mM NaCl, 2 mM $MgCl_2$, 0.22 mM EGTA, 1 mM Hepes, pH 7). After 15 min on ice 2 mM ATP or AMP-PNP were added and tonicity was restored by addition of 900 mM KCl, 450 mM NaCl, 20 mM $MgCl_2$, 2 mM EGTA, 100 mM Hepes, pH 7, and cells were resealed for 1 h at 37 °C. Resealed ghosts were collected by centrifugation (10 min, 3,200 $\times$ *g*) and washed three times with buffer A.

Metabolic Labeling—Fibroblasts in 6-well plates were preincubated for 20 min with 1 μg/ml brefeldin A to block vesicular traffic, with or without candidate translocation inhibitors. The cells were labeled for the indicated times with 20 μCi/well [^{32}P]phosphate (about 9×10^5 cells), washed with PBS, and chased for different time periods before analysis with a cell surface PC assay. Erythrocytes were preincubated for 20 min with or without inhibitors, labeled with trace amounts of [^{14}C]arachidonic, -oleic, or -palmitic acid (0.1–1 nmol/100 nmol of erythrocyte lipids) or with [^{14}C]palmitoylcarnitine (1.5 nmol/100 nmol of erythrocyte lipids), washed with 2% BSA and twice with buffer A, and chased for the indicated time periods with or without inhibitors before analysis with a cell surface PC assay.

PLA_2 Cell Surface Assay—Cells (150 nmol of phospholipid) were incubated in 500 μl of buffer A plus 10 mM $CaCl_2$ and 50 IU bee venom phospholipase A_2 (PLA_2) for 5 min at 37 °C. Hemolysis as the absorption of the cell supernatant at 540 nm in a spectrophotometer was below 4%. Lipids were quantified after two-dimensional TLC analysis. [^{14}C]PC exposure at the erythrocyte surface was expressed as % decrease of [^{14}C]PC in the PLA_2-treated sample as compared with the identically treated control but without PLA_2. If [^{14}C]PC in the PLA_2-treated sample was not decreased but slightly higher than in the control, this way of quantification generates negative values. Alternatively, [^{14}C]PC exposure at the erythrocyte surface was expressed as % of 1-[^{14}C]palmitoyl lyso-PC of the total 1-[^{14}C]palmitoyl-PC in the control without PLA_2. For the quantification of total 1-[^{14}C]palmitoyl-PC in the control, lipids were extracted, dried under nitrogen, and dissolved in 10 μl of diethyl ether, added to 1 ml of buffer A plus 10 mM $CaCl_2$, containing 1% BSA and 20 IU PLA_2, and incubated for 30 min at 37 °C, after which 1-[^{14}C]palmitoyl lyso-PC was quantified. In fibroblasts, [^{32}P]PC at the cell surface was measured as % of [^{32}P]lyso-PC in the PLA_2-treated sample from total [^{32}P]PC ([^{32}P]lyso-PC + [^{32}P]PC) in the control without PLA_2.

PC Transfer Protein (PCTP) Cell Surface Assay—PCTP (a kind gift from K. Wirtz (CBLE, Utrecht)) (39), was stored in 50% glycerol, which was removed before the assay by using Centricon YM 10 filter units (Millipore, Etten-Leur, The Netherlands). For investigating the outward translocation of [^{14}C]PC, small unilamellar vesicles (SUVs) were generated by sonication on ice (18) of egg PC:cholesterol:egg PS (50:50:1, mol/mol). The amount of SUVs in the cell pellet was below 2% as measured by [^{14}C]cholesterol ester as a non-exchangeable SUV marker. Cells containing 100 nmol of phospholipid were labeled for 40 min with [^{14}C]arachidonate, washed with buffer A, 2% BSA, and twice with buffer A and incubated at 37 °C in 500 μl of buffer A, 15 mM glucose, 3 nmol of PCTP, a 10-fold excess of SUV PC, and a 20-fold excess of cold arachidonate under slow rotation. After the incubation, the cells were washed three times in buffer A containing 1% glycerol. The supernatants were pooled and the lipids of cells and supernatants were analyzed. Cell surface [^{14}C]PC was quantified as [^{14}C]PC in the PCTP supernatant as % of total [^{14}C]PC in supernatant plus cells. In [^{14}C]PC inward translocation studies, SUVs were generated by sonication of (*a*) PC isolated from [^{14}C]arachidonate-labeled erythrocytes:cholesterol: egg PS (50:50:1) or (*b*) [^{32}P]PC isolated from mouse fibroblasts:cholesterol:egg PS (50:50:1). Erythrocytes containing 100 nmol of phospholipid were incubated at 37 or 4 °C in 500 μl of buffer A, 15 mM glucose, 3 nmol of PCTP and SUVs. The ratio of total erythrocyte PC to SUV PC was between 1:10 and 1:2. After the incubation, the erythrocytes were washed 3 times with buffer A, 1% glycerol before further incubation and a PLA_2 assay.

Outward Translocation of C_6-NBD-PC—C_6-NBD-PC cell surface

TABLE I
Phospholipid hydrolysis in intact human erythrocytes by phospholipase A_2

	% Surface exposure			
	PLA_2	PC	PE	PS
	min at 37 °C			
Total phospholipids[a]	5	61 ± 1	12 ± 4	0 ± 14
Total phospholipids[b,c]	60 (120)	68 (76)	0–20 (20)	0
Total phospholipids[d]	60	55	9	0
[^{32}P]PC inserted at 4 °C[e]	5	92 ± 1		
[^{14}C]Arachidonate-labeled phospholipids[a,f]	5	56 ± 7	28 ± 8	4 ± 20
[^{14}C]Oleate-labeled phospholipids[a,f]	5	53 ± 6	32 ± 19	ND[g]
[^{14}C]Palmitate-labeled phospholipids[a,f]	5	52 ± 6	28 ± 13	ND

[a] PLA_2 cell surface assay in this study: 50 IU bee venom PLA_2 in 0.5 ml of buffer A containing 10 mM $CaCl_2$, for 50 μl of packed human erythrocytes, containing approximately 150 nmol of total phospholipid, 42 nmol of PC, 46 nmol of PE, 39 nmol of sphingomyelin, and 17 nmol of PS.
[b] Results from the literature with 5–10 IU *Naja naja* PLA_2 and 250 μl of packed erythrocytes in 5 ml for 1 h (1).
[c] Results from the literature with PLA_2 for 1 h followed by PLA_2 + sphingomyelinase for 1 h (2).
[d] Results from the literature with bee venom PLA_2 for 1 h (2).
[e] [^{32}P]PC was inserted from liposomes at 4 °C as described under "Experimental Procedures," followed by hydrolysis by PLA_2.
[f] Human erythrocytes were pulse-labeled with trace amounts [^{14}C]arachidonic, [^{14}C]oleic, and [^{14}C]palmitic acid, washed twice with 2% BSA, and chased overnight in buffer A, 15 mM glucose. The distribution of the labeled lipids across the membrane was investigated with the PLA_2 assay and expressed as % decrease of [^{14}C]PC in the PLA_2 sample from [^{14}C]PC in the control (mean ± S.D., $n = 3$).
[g] ND, not determined.

translocation was measured essentially as described by Connor *et al.* (15). 1 ml of packed human erythrocytes were incubated for 90 min at 37 °C with 15–20 nmol of C_6-NBD-PC in buffer A, 15 mM glucose, containing 1 M ethanol to reversibly accelerate phospholipid flip (40). Because all samples were identically labeled with C_6-NBD-PC in the presence of 1 M ethanol, differences in translocation rates are irrespective of the ethanol. C_6-NBD-PC still at the cell surface was removed by two washes with 20 volumes of buffer A, 2% BSA, for 2 min at room temperature. Outward translocation of the lipid analog was measured by incubating the cells at 37 °C in buffer A, 15 mM glucose, with or without candidate inhibitors for various times. Cell surface C_6-NBD-PC was depleted with BSA as described above. Lipids in the cells and BSA washes were extracted and separated by one-dimensional TLC in acidic solvent. C_6-NBD-PC cell surface exposure was measured as % of C_6-NBD-PC in the supernatant from total C_6-NBD-PC in cells plus supernatant. % C_6-NBD-FA was below 10% of total NBD fluorescence and increased during the chase by less than 5%, independent of the inhibitors.

RESULTS

To investigate the outward translocation of natural PC across the plasma membrane of mammalian cells, we first optimized two independent PC cell surface assays in erythrocytes. Here, the absence of intracellular membranes facilitates the analysis of transport processes at the plasma membrane and allows to optimally control cell surface assay conditions. If the assay induced disturbances at the plasma membrane, these would be detectable by changes in total lipid asymmetry, erythrocyte shape, and by increased hemolysis. The high percentage of PC at the erythrocyte surface is favorable for its quantification.

Assays for the Translocation of Natural PC

Generation of Labeled PC at the Inside of the Plasma Membrane—The investigation of the outward translocation of natural PC depends on placing a labeled PC at the inside of the cell and an assay for its appearance on the outside. Mature erythrocytes are not able to synthesize lipids *de novo* but exogenous fatty acids are rapidly taken up and coupled to pre-existing lyso-PC via an ATP-dependent acyl-CoA synthetase and an acyl-CoA:lysophospholipid acyltransferase at the inside of the plasma membrane (24–26). The sidedness of PC labeling was demonstrated experimentally. Insertion of 5 mol % of lyso-PC into the outer leaflet of the erythrocyte membrane enhanced the incorporation of [^{14}C]arachidonic acid into PC, but not into PE or PS. This effect was only seen after increasing chase periods and was after 5 min of chase 111%, after 15 min 140%, and after 25 min 203% of PC labeling in the control without exogenous lyso-PC. In contrast, when lyso-PC was offered to both sides of the plasma membrane during hypo-osmotic shock, PC labeling increased to 2300% after 15 min, supporting bulk PC labeling at the inside. The stimulatory effect of exogenous lyso-PC on PC labeling in intact erythrocytes is best explained by slow inward movement of lyso-PC.

Detecting Labeled PC at the Cell Surface—Two methods have been used to measure the transbilayer distribution of PC across the erythrocyte membrane, hydrolysis of surface PC by PLA_2 (1, 24–26) and exchange of outer leaflet PC against liposomal PC by a lipid transfer protein (28, 41).

First, we optimized the PLA_2 assay to reduce incubation times and allow the immediate detection of newly synthesized PC at the cell surface. Incubation of a 50-μl erythrocyte pellet with 50 IU PLA_2 in 500 μl of buffer A, 10 mM $CaCl_2$ for 5 min at 37 °C resulted in almost complete degradation of cell surface PC, namely 92 ± 1.4% of [^{32}P]PC that had been introduced into the outer bilayer leaflet by PCTP at 4 °C (Table I). The generated degradation pattern of total phospholipid was in agreement with the literature on erythrocyte lipid asymmetry. No breakdown of PS was detected, demonstrating restriction of PLA_2 activity to the surface lipids, unless 1% BSA was present with PLA_2, causing hemolysis by extracting free fatty acids and lysolipids. The PLA_2 hydrolysis pattern of the [^{14}C]fatty acid-labeled phosphoglycerolipids (Fig. 1) largely reflected that of total lipids, showing full equilibration of the labeled lipids after overnight chase. When analyzing the cell surface exposure of [^{14}C]arachidonoyl-PC and [^{14}C]oleoyl-PC after 30 min labeling, we already found 37 ± 4 and 28 ± 17% at the cell surface, which increased to 56 ± 7 and 53 ± 6% after an overnight chase, respectively, suggesting fast outward translocation of PC with a half-time below 30 min. Also [^{14}C]PE rapidly appeared at the cell surface, however, the large standard deviations inherent to the determination of the minor fractions of PE at the cell surface hampered more precise quantifications (Table I).

As an independent assay for cell surface PC we used the PC-specific transfer protein PCTP. PCTP stimulates the exchange of single PC molecules between membrane surfaces, has minimal perturbing effects on membrane structure, and has been used for investigating the size of the surface PC pool (28). The suitability of PCTP for measuring PC outward translocation in erythrocytes has not been demonstrated before. The PCTP assay did not result in hemolysis for up to 4 h, and still only cell surface lipids were available to PLA_2 (not shown). After labeling erythrocytes with [^{14}C]arachidonate for 30 min,

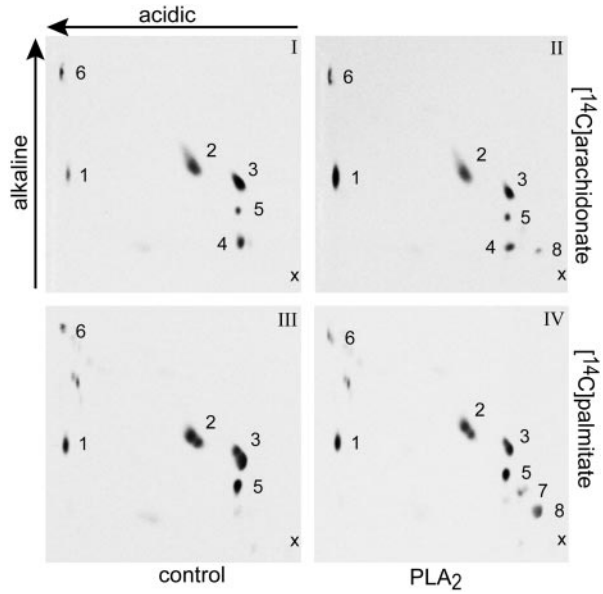

FIG. 1. **The PLA_2 cell surface assay.** Erythrocytes were labeled overnight with [^{14}C]arachidonic acid (*I* and *II*) or [^{14}C]palmitic acid (*III* and *IV*) and treated with PLA_2 (*II* and *IV*) or the buffer without the enzyme (*I* and *III*) for 5 min at 37 °C. Lipids were extracted, separated by two-dimensional TLC, and analyzed by a phosphorimager. The PLA_2 treatment of [^{14}C]arachidonate-labeled cells yielded a loss of [^{14}C]PC and production of free [^{14}C]arachidonic acid, whereas PLA_2 treatment of [^{14}C]palmitate-labeled cells produced a significant amount of [^{14}C]lyso-PC as well (*1*, free fatty acid; *2*, PE; *3*, PC; *4*, PS; *5*, acylcarnitine; *6*, non-polar lipids; *7*, lyso-PE; *8*, lyso-PC; *x*, origin).

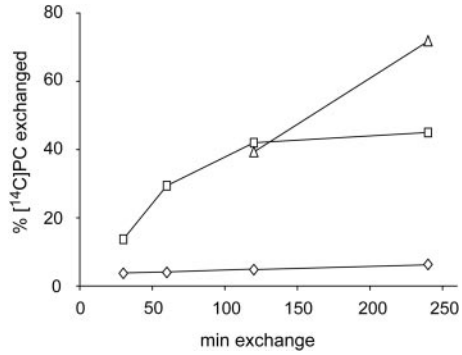

FIG. 2. **The PCTP assay for cell surface PC.** Erythrocytes were steady state labeled with [^{14}C]arachidonic acid and incubated with PCTP at 37 °C (33 nmol of total erythrocyte PC, 330 nmol of PC in SUVs with 3 nmol of PCTP (□), or with 3 nmol of BSA (◇). In △, the PCTP containing supernatant was removed at 2 h and replaced by fresh PCTP and SUVs.

36 ± 5% of the [^{14}C]PC was transferred to liposomes during a subsequent 2-h incubation with PCTP. This increased to 46 ± 7% when the PCTP assay was performed after an overnight chase, which is in the same range as the [^{14}C]PC cell surface exposure detected by the PLA_2 assay. However, the addition of fresh liposomes and PCTP after the 2-h PCTP incubation enhanced [^{14}C]PC exchange with the unlabeled liposomes, showing that the transfer was not complete after 2 h and suggesting that PC outward translocation was fast on this time scale (Fig. 2). Indeed, a PLA_2 cell surface assay subsequent to the PCTP exchange still localized 30% of the remaining [^{14}C]PC at the surface.

Time Course of 1-[^{14}C]Palmitoyl-PC Cell Surface Translocation

To investigate the time course of [^{14}C]PC outward translocation by a pulse-chase approach (26), we analyzed the cell surface exposure of [^{14}C]oleoyl-PC after short labeling periods by using PLA_2. In contrast to the previous study by Andrick *et al.* (26), we noticed that during the PLA_2 assay, synthesis of [^{14}C]PC continued and was much higher than in the control incubation without PLA_2. After 5 min labeling, the incorporation of [^{14}C]oleic acid into [^{14}C]oleoyl-PC was 149 ± 37% in the PLA_2-treated sample as compared with the control. A small fraction of the PLA_2-produced lyso-PC must have translocated to the inner leaflet where it was very efficiently ^{14}C-acylated, as we had observed for exogenous lyso-PC. Because [^{14}C]oleic acid or [^{14}C]arachidonic acid is predominantly incorporated in the S_n2 position, [^{14}C]PC cell surface exposure has to be calculated by the loss of [^{14}C]PC in the PLA_2-treated sample as compared with the control. The lyso-PC-induced increase of [^{14}C]PC in the PLA_2 sample would yield a dramatic underestimate of [^{14}C]PC translocation: full equilibration of [^{14}C]oleoyl-PC between the two leaflets after 5 min labeling would be scored as ~25% instead of 50% translocation.

To avoid this interference, we decided to use in short term labeling experiments the production of 1-[^{14}C]lyso-PC instead of the loss of [^{14}C]PC. For this we applied [^{14}C]palmitate, which efficiently labels the S_n1 position of PC and is therefore retained in lyso-PC (Fig. 1). The cell surface exposure of 1-[^{14}C]PC was determined as the amount of 1-[^{14}C]palmitoyl lyso-PC in the PLA_2-treated sample as % of the total 1-[^{14}C]palmitoyl-PC in the control sample (see "Experimental Procedures"). Re-acylation of labeled 1-[^{14}C]palmitoyl lyso-PC would decrease the amount of 1-[^{14}C]palmitoyl lyso-PC in the PLA_2-treated sample. The increase in [^{14}C]PC during the PLA_2 assay allowed us to estimate that maximally 2–3% of the produced lyso-PC flipped from the outer leaflet back into the inner leaflet during the PLA_2 assay, which has minimal effects on the calculation of the cell surface exposure of 1-[^{14}C]PC.

After a 5-min labeling with [U-^{14}C]palmitic acid and chase periods in the range of minutes we found a time-dependent increase in the percentage of 1-[^{14}C]PC at the surface (Fig. 3) and concluded an initial rate of translocation of 0.7% [^{14}C]PC per min. Non-linear regression of the data resulted in half-times of equilibration of 28 min resulting in a maximal level of 1-[^{14}C]palmitoyl-PC at the cell surface of 36 ± 5% after an 18-h chase. Assuming that the PLA_2 treatment would inhibit translocation of newly synthesized PC (26), the half-time for the 1-[^{14}C]palmitoyl-PC outward equilibration would be even shorter. For total [^{14}C]palmitoyl-PC, [^{14}C]oleoyl-PC, and [^{14}C]arachidonoyl-PC this fraction was 52 ± 6, 53 ± 6, and 56 ± 7%, respectively (Table I). The lower cell surface availability of 1-[^{14}C]palmitoyl-PC suggests a preference of this PC species for the inner leaflet of the membrane. A preferential localization of palmitate-labeled as compared with arachidonate-labeled PC at the inside of the human erythrocyte membrane was reported by Renooij *et al.* (24).

Energy Dependence of PC Cell Surface Translocation

To test the energy dependence of [^{14}C]PC outward translocation we reduced the erythrocyte ATP levels by preincubating the cells for 2 h with deoxyglucose and KF, which reduced erythrocyte ATP levels by 80–90% (37). In energy-depleted cells [^{14}C]palmitate is preferentially incorporated into the PC from [^{14}C]palmitoyl-L-carnitine (42) that is translocated across the erythrocyte membrane in an energy-independent way with a half-time of 2.6 h (43) and was used for metabolic labeling of PC. After 2 h labeling with [^{14}C]palmitoyl-L-carnitine only, 8 ± 12% (n = 4) of the labeled PC was accessible to PLA_2 at the surface of energy-depleted cells as compared with 36 ± 11% (n = 4) in the fresh erythrocytes, demonstrating that [^{14}C]PC translocation is energy-dependent. Because the formation of [^{14}C]PC from [^{14}C]palmitoyl-L-carnitine was 297 ± 29% in en-

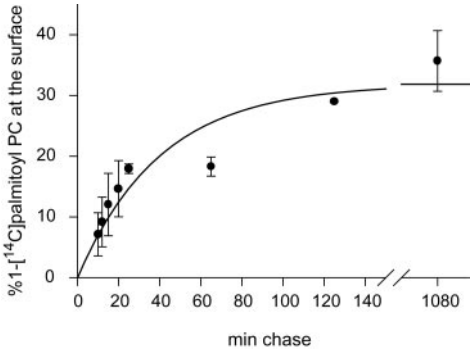

FIG. 3. **Time course of 1-[^{14}C]palmitoyl-PC cell surface translocation.** Fresh erythrocytes were labeled for 5 min with [U-^{14}C]palmitic acid, washed, and chased for the indicated times at 37 °C followed by the PLA_2 cell surface assay. Cell surface exposure is expressed as % 1-[^{14}C]palmitoyl-PC digested. Total 1-[^{14}C]palmitoyl-PC was determined by completely digesting the isolated lipids of a parallel sample with PLA_2 (mean ± S.D., $n = 5$). For the various time points, 1-[^{14}C]palmitoyl-PC made up 57 ± 6% ($n = 5$) of the total [^{14}C]palmitoyl-PC.

ergy-depleted cells as compared with fresh erythrocytes, one could argue that the absolute amount of [^{14}C]PC translocated under both conditions was the same. However, when PC in energy-depleted cells was labeled with [^{14}C]arachidonic acid, which is less efficiently incorporated into PC in energy-depleted cells as compared with fresh erythrocytes, we found a similar reduction in the cell surface exposure of labeled PC in energy-depleted cells with the PLA_2 and PCTP cell surface assays (Fig. 4*A*).

If the reduced PC cell surface translocation was the direct result of ATP depletion, restoration of ATP levels should restore PC translocation. For this, we prepared ghosts from labeled, energy-depleted erythrocytes, resealed them in the presence or absence of ATP or its non-hydrolysable analog AMP-PNP, and investigated the exposure of [^{14}C]PC at the cell surface by PLA_2. In the resealed ghosts, ATP but not the non-hydrolysable AMP-PNP restored [^{14}C]PC surface translocation (Fig. 4*B*). The effective ATP concentration in the resealing buffer was in the millimolar range in accordance with the physiological erythrocyte ATP concentration (Fig. 4*C*). There was no major difference in the phospholipid asymmetry of fresh and energy-depleted erythrocytes or the corresponding ghosts, in accordance with the data in the literature (44) (Table II).

PC Cell Surface Translocation: A Role for ABC Transporters?

Recently, the family of ABC transporters has been associated with lipid homeostasis and the multidrug resistance proteins ABCB1, ABCB4, and ABCC1 have been shown to translocate short chain lipids to the cell surface (45). Because a number of ABC transporters have been found in erythrocytes, we tested the effect of various established inhibitors on the translocation of C_6-NBD-PC, and subsequently, [^{14}C]PC (Fig. 5, *A* and *B*). MK571, PSC833, and glibenclamide reduced the outward translocation of C_6-NBD-PC by over 50% as demonstrated before in erythrocytes for various inhibitors of multidrug transporters (23, 46). 0.2 μM Ko143, which inhibits the multidrug transporter ABCG2 (47), had no effect unless used at a high concentration (10 μM) where it probably affected ABCB1 and ABCC1 (48). Vanadate, a general inhibitor of P-type ATPases, almost abolished cell surface translocation of C_6-NBD-PC (Fig. 5*A*). As for C_6-NBD-PC, cell surface translocation of natural PC was reduced by glibenclamide and vanadate, both in a PLA_2 and PCTP assay (Fig. 5*B*). In contrast, PSC833 and MK571, which effectively reduced the translocation of C_6-NBD-PC, had no effect on the surface exposure of [^{14}C]PC (not shown).

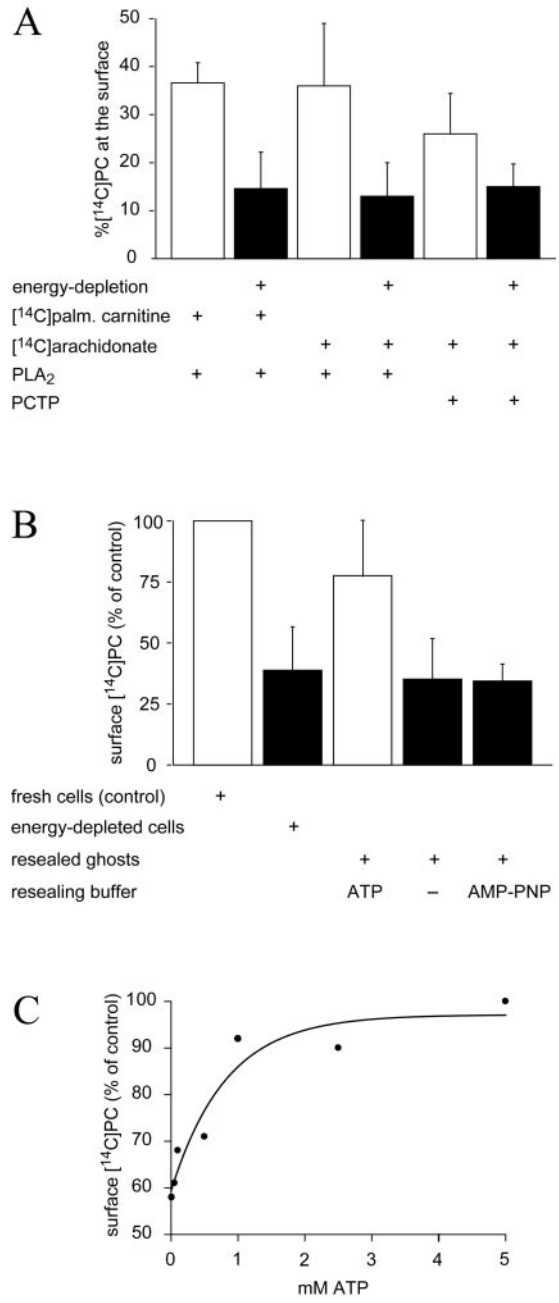

FIG. 4. **ATP dependence of 1-[^{14}C]PC outward translocation in human erythrocytes and resealed erythrocyte ghosts.** *A*, erythrocytes were preincubated for 2 h with 50 mM deoxyglucose and 5 mM KF, labeled in energy-depletion buffer with [^{14}C]palmitoylcarnitine ($n = 3$) or [^{14}C]arachidonic acid for 2 h ($n = 2$ for both experimental conditions), and subjected to a PLA_2 or PCTP cell surface assay without glucose. In parallel, fresh erythrocytes were labeled and assayed under normal conditions. Cell surface exposure is expressed as % of cell surface 1-[^{14}C]palmitoyl-PC as in Fig. 3 or as % decrease of [^{14}C]arachidonoyl-PC in the PLA_2 sample from [^{14}C]arachidonoyl-PC in the control. In the PCTP assay [^{14}C]PC cell surface exposure is expressed as % of [^{14}C]PC in the PCTP supernatant from total [^{14}C]PC in supernatant plus cells (mean ± S.D.). *B*, 1-[^{14}C]palmitoyl-PC cell surface exposure was investigated by PLA_2 in [^{14}C]palmitoylcarnitine labeled fresh ($n = 3$) and energy-depleted erythrocytes ($n = 3$) and in ghosts of energy-depleted erythrocytes that were resealed for 1 h at 37 °C in the presence of buffer alone ($n = 4$), buffer with 2 mM ATP ($n = 7$), or with 2 mM AMP-PNP ($n = 3$). Data (mean ± S.D.) are related to the cell surface exposure of 1-[^{14}C]palmitoyl-PC in fresh erythrocytes. *C*, ghosts from [^{14}C]palmitoylcarnitine-labeled energy-depleted erythrocytes were resealed for 1 h at 37 °C in the presence of the indicated ATP concentration. Data are presented as % of the maximal 1-[^{14}C]palmitoyl-PC cell surface exposure of the experiment with 5 mM ATP, where 25% 1-[^{14}C]palmitoyl-PC was at the surface.

TABLE II
Cell surface exposure of total glycerophospholipids in fresh and energy-depleted human erythrocytes and resealed ghosts

	% Surface exposure[a]		
	PC	PE	PS
Fresh erythrocytes	57 ± 5	9 ± 3	6 ± 8
Energy-depleted erythrocytes	58 ± 0	29 ± 7	12 ± 23
Ghosts resealed with 2 mM ATP[b]	57 ± 6	25 ± 1	15 ± 9
Ghosts resealed without ATP[b]	56 ± 2	25 ± 8	15 ± 16

[a] Fresh and energy-depleted erythrocytes and resealed ghosts of the latter were subjected to the PLA_2 assay (experiments in Fig. 4*B*), and the hydrolysis of the total glycerophospholipids was determined by phosphate determination (mean ± S.D., $n = 2$).
[b] Resealed ghosts from energy-depleted erythrocytes.

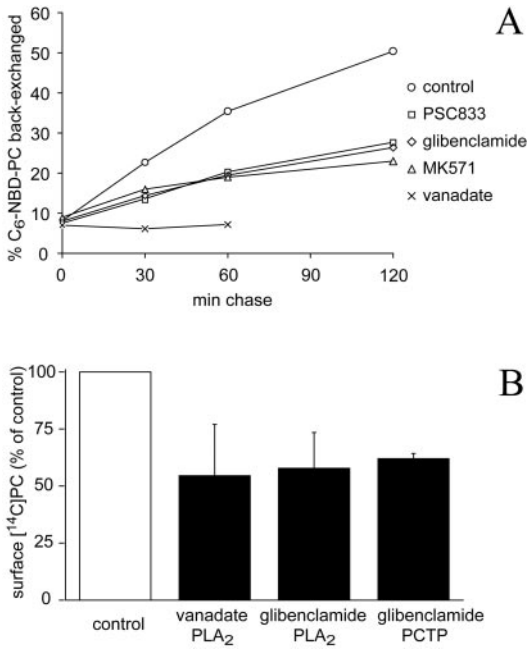

FIG. 5. **Inhibitors of PC cell surface translocation in human erythrocytes.** *A*, after labeling the inner leaflet of the human erythrocyte membrane with C_6-NBD-PC, the cells were incubated at 37 °C with or without different inhibitors (50 μM glibenclamide, 5 μM PSC833, 5 μM MK571, 1 mM vanadate). After increasing time periods aliquots were taken and cell surface C_6-NBD-PC was measured by BSA back-exchange. *B*, erythrocytes were preincubated for 20 min with or without 50–100 μM vanadate (PLA_2, $n = 8$) or 50 μM glibenclamide (PLA_2, $n = 10$ and PCTP, $n = 3$) and labeled with [^{14}C]palmitate and [^{14}C]arachidonate for 1 h before analysis in the PLA_2 or PCTP assay. Data are presented as percent of control without inhibitor (mean ± S.D.).

To test whether individual ABC transporters were involved in natural PC translocation, we studied the cell surface appearance of [^{14}C]PC in erythrocytes from available ABC transporter knockout mice by using PLA_2 (Table III). Investigations on erythrocytes from triple Abcb1a/1b$^{-/-}$, Abcc1$^{-/-}$, double Abcb1a/1b$^{-/-}$ and Abcb4$^{-/-}$ knockout mice, and their controls revealed that the [^{14}C]PC cell surface exposure was in average 59, 63, and 40% of that in the matched controls, suggesting a possible involvement of these transporters in PC translocation. In contrast, we observed no decrease in [^{14}C]PC cell surface exposure in Abcc7$^{-/-}$ mice as compared with the heterozygous controls. (The investigation of erythrocytes from Abca1$^{-/-}$ animals by PLA_2 was obscured by increased hemolysis (49).)

PC Inward Translocation

In view of the relatively fast cell surface translocation of natural PC, we would expect a compensatory PC flux in the inward direction to maintain the steady state plasma membrane PC distribution. To investigate inward translocation of

TABLE III
Cell surface exposure of labeled and total phospholipids in erythrocytes from ABC transporter knockout mice

	% Surface exposure[a]			
	[^{14}C]PC	[^{14}C]PE	Total PC	Total PE
Control[b]	32 ± 5	19 ± 3	36 ± 7	11 ± 3
Abcb1a/1b, Abcc1$^{-/-b}$	19 ± 12	21 ± 10	35 ± 3	9 ± 4
Control[c]	24 ± 4	ND[d]	45[e]	18[e]
Abcb1a/1b$^{-/-c}$	15 ± 3	ND	45[e]	10[e]
Control[b]	34 ± 6	23 ± 8	34 ± 1.2	9 ± 1.3
Abcb4$^{-/-b}$	13 ± 10	22 ± 19	32 ± 1.8	12 ± 0.8
Abcc7$^{+/-b}$	28 ± 11	35 ± 8	41 ± 3	11 ± 4
Abcc7$^{-/-b}$	32 ± 9	38 ± 12	44 ± 2	15 ± 8

[a] Mouse erythrocytes were labeled with [^{14}C]arachidonic or [^{14}C]palmitic acid for 90 min and investigated for cell surface exposure of [^{14}C] and total phospholipids by PLA_2 (mean ± S.D., $n = 3$). In accordance with the literature where 50–60% of total PC were exchanged by PCTP in mouse (76) as compared to 75% in human cells (28), we found with PLA_2 less PC at the surface of mouse erythrocytes: 39 ± 5% as compared to 61 ± 1% in the human cells (Table I).
[b] % of [^{14}C]Arachidonoyl-PC surface exposure.
[c] % of 1-[^{14}C]Palmitoyl-PC surface exposure.
[d] ND, not determined.
[e] Single determinations.

PC, the erythrocyte surface was first loaded with labeled PC using PCTP (7). After a 2-h exchange at 37 °C with SUVs containing [^{14}C]arachidonoyl-PC, only 65 ± 4% of the labeled PC was degradable at the surface by PLA_2 ($n = 6$). However, when the labeling was performed at 4 °C almost all exchanged labeled PC was hydrolyzed (92 ± 1%, $n = 6$), suggesting that the temperature dependent formation of a PLA_2 protected [^{14}C]PC pool reflects PC inward translocation. After 2 h at 37 °C [^{14}C]arachidonoyl-PC had almost reached its steady distribution where 61 ± 1% were degraded in a PLA_2 cell surface assay (Table I).

PC Cell Surface Translocation in Nucleated Cells

[^{32}P]PC at the Surface of Mouse Fibroblasts—To see whether the active outward PC translocation observed in erythrocytes is a general feature of mammalian cells, we labeled PC in mouse fibroblasts with [^{32}P]phosphate and measured its appearance at the surface by using PLA_2. Incubation conditions that degrade 61 ± 1% of the erythrocyte PC (50 IU PLA_2, 5 min) only degraded 8.9 ± 0.2% of fibroblast [^{32}P]PC after 2 h of labeling. Higher concentrations of PLA_2 and overnight [^{32}P]phosphate labeling did not significantly increase [^{32}P]PC degradation (2 h of [^{32}P]phosphate/5 min, 100 IU PLA_2: 10.1 ± 0.8% [^{32}P]PC; 20 h of [^{32}P]phosphate/5 min, 50 IU PLA_2: 8.3 ± 3.2% [^{32}P]PC). Thus, either 9% of the [^{32}P]PC had been degraded in every fibroblast, or, alternatively, the fibroblasts were insensitive to PLA_2 and 9% of the fibroblasts had lysed and had their complete [^{32}P]PC content degraded. It is known that exposure of PLA_2-treated erythrocytes to BSA, which extracts fatty acids and lysolipids, leads to membrane destabilization and lysis. Thus, if PLA_2 had degraded the surface phospholipids in every fibroblast, they should all be lysed by BSA. Addition of BSA during the PLA_2 digest tremendously increased the hydrolysis of labeled PS, an intracellular lipid, from virtually zero to 70 ± 3.5%, showing that BSA lysed at least 70% but probably all of the fibroblasts. Thus, in all fibroblasts the surface lipids had been hydrolyzed by PLA_2 and only 9% of the cellular PC was present on the cell surface.

Transport of Newly Synthesized PC to the Surface of Mouse Fibroblasts—In nucleated cells PC is synthesized on the cytoplasmic side of the ER and equilibrates with PC on the lumenal side by an as yet unidentified energy-independent "flippase." From here, PC can reach the cell surface by vesicle traffic. To investigate the existence of a vesicle independent pathway of

TABLE IV
Newly synthesized PC reaches the cell surface in the absence of vesicular trafficking

	Labeling (MF cells)[a]		
	30 min	90 min	120 min
% of [^{32}P]PC at the cell surface, no BFA	2.4 ± 0.6	4.3 ± 0.1	8.0 ± 0.2
% of [^{32}P]PC at the cell surface, 1 μg/ml BFA	1.8 ± 0.6	4.4 ± 0.4	7.7 ± 0.2

[a] Mouse fibroblasts (wt1.2) were preincubated for 20 min in the presence or absence of 1 μg/ml BFA, labeled for the indicated times with [^{32}P]phosphate, and washed with buffer A before addition of 100 IU PLA_2 for 5 min (mean ± S.D., $n = 2$).

PC transport to the surface, mouse fibroblasts were labeled in the presence of 1 μg/ml brefeldin A (BFA), which blocks the secretory pathway (50). BFA strongly reduced the surface exposure of newly synthesized sphingomyelin, which is synthesized in the Golgi lumen and reaches the cell surface exclusively via vesicle transport (51). However, the arrival of newly synthesized PC at the cell surface was unaffected by BFA (Table IV), showing the existence of a non-vesicular pathway through the cytosol and subsequent transbilayer translocation. Next, we wanted to investigate the time course of PC cell surface appearance. Because we were unable to efficiently stop [^{32}P]PC synthesis and to perform a pulse-chase, we measured the time course of [^{32}P]PC cell surface appearance in the presence of the labeled substrate. We found a gradual increase of newly synthesized PC at the cell surface with time to roughly 10% after 2 h (Fig. 6*A*). Cytosolic transport of newly synthesized PC to the plasma membrane is supposed to be fast and to occur in minutes (52). However, the rate of [^{32}P]PC cell surface appearance is an underestimate of the true rate of PC translocation, because transport was studied under conditions of ongoing synthesis.

Inhibitors of PC Cell Surface Translocation in Mouse Fibroblasts—As in erythrocytes, glibenclamide reduced the cell surface exposure of [^{32}P]PC by 40–50%. This could be demonstrated by the PLA_2 and PCTP cell surface assays (Fig. 6, *A* and *B*). The decreased percentage of newly synthesized PC at the cell surface was not a result of increased ^{32}P labeling. In contrast, glibenclamide decreased PC synthesis (*inset* of Fig. 6*A*). A similar sensitivity toward glibenclamide was also seen for [^{32}P]PE cell surface translocation. However, the differences were statistically not significant (data not shown). The cell surface exposure of newly synthesized PC was also reduced by vanadate as demonstrated by a PCTP PC cell surface assay (Fig. 6*B*), whereas transport of PC from its site of synthesis in the endoplasmic reticulum to the plasma membrane was energy-independent (52). Although we cannot directly discriminate which process was affected by the inhibitors, intracellular transport or plasma membrane translocation, the similarity with the effect in erythrocytes suggests a common target of glibenclamide and vanadate action and a related or identical molecular mechanism of PC translocation in the two cell types.

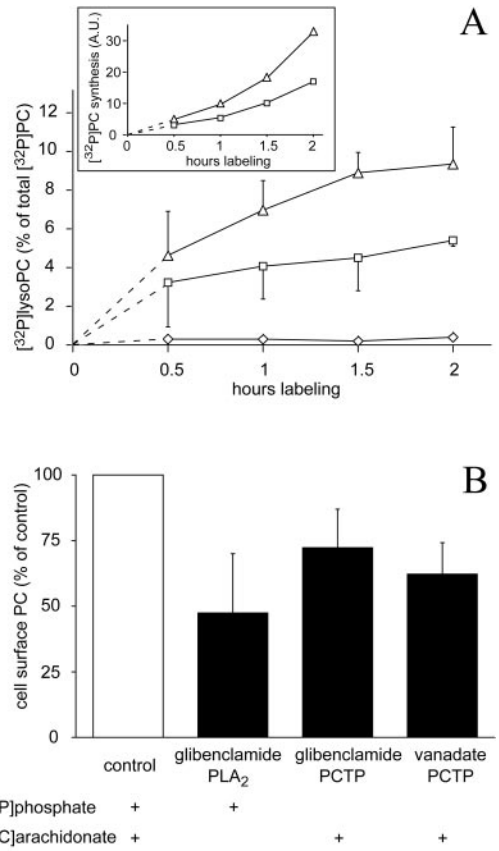

FIG. 6. **Appearance of [^{32}P]PC at the cell surface of fibroblasts.** *A*, Wt1.2 mouse fibroblasts were preincubated with BFA in the absence (△) or presence of 50 μM glibenclamide (□) and labeled with [^{32}P]phosphate. After increasing labeling periods the cells were treated with PLA_2 (mean ± S.D., $n = 4$) or the buffer without the enzyme (◇). The *inset* shows [^{32}P]PC synthesis in fibroblasts in a representative experiment. *B*, fibroblasts were preincubated for 20 min with BFA in the presence or absence of 50 μM glibenclamide ($n = 9$), labeled for 90 min with [^{32}P]phosphate, and investigated for [^{32}P]PC cell surface exposure by PLA_2. Alternatively, cells were preincubated with BFA in the presence or absence of 50 μM glibenclamide ($n = 2$) or 1 mM vanadate ($n = 3$), labeled for 1 h with [^{14}C]arachidonic acid, and were investigated for [^{14}C]PC cell surface exposure by PCTP. Data are expressed as % of the control without inhibitor (mean ± S.D.).

DISCUSSION

Active Cell Surface Translocation of Endogenous PC—In the present study, we have characterized the outward translocation of natural PC in human and mouse erythrocytes and mouse fibroblasts. Previous investigations demonstrated that newly synthesized PC rapidly appeared at the cell surface of rat (25) and human erythrocytes (26) by using PLA_2. After uncovering unexpected complexities in the application of PLA_2 after short term pulse labeling, we redesigned the PLA_2 assay for the investigation of PC cell surface translocation and confirmed our main results by an independent PCTP cell surface assay. In earlier studies, fast translocation was specific for PC, appeared energy independent by its vanadate insensitivity, and directly coupled to PC synthesis. In contrast, we observed rapid cell surface translocation of both PC and PE and show that PC translocation was energy-dependent and involved bulk PC.

PC cell surface translocation was strongly reduced in energy-depleted erythrocytes and was restored by re-addition of hydrolysable ATP, but not AMP-PNP. In fresh erythrocytes, 50 μM vanadate, an inhibitor of P-type ATPases and ABC transporters, reduced PC cell surface translocation. This energy dependence excluded the possibility that the cell surface exposure of [^{14}C]PC was mediated by the ATP-independent PC translocation activity described in membranes of rat liver (but not erythrocytes and other non-hepatic cells) (27, 53) or by the energy-independent, calcium-activated scramblase (54), which was further excluded by (i) the absence of extracellular calcium during labeling and chase; (ii) the lack of effect of DIDS (50 μM), a scramblase inhibitor (55) (data not shown); and (iii) by the fact that the total plasma membrane phospholipid distribution remained normal. The effect of vanadate on [^{14}C]PC cell surface translocation may have been missed in previous studies using high inhibitor concentrations (1 mM) (26) because of secondary effects. We found that increasing concentrations of

vanadate induced the formation of echinocytes, suggesting redistribution of membrane phospholipids with an expansion of the outer bilayer leaflet, which in combination with PLA_2 could affect membrane integrity. ATP dependence and vanadate sensitivity of PC outward translocation were so far only demonstrated for short chain PC analogs in the case of ABCB4 and the highly homologous multidrug transporter ABCB1 in cells (19) and proteoliposomes (56) and for ABCC1 in human erythrocytes (57) and proteoliposomes (58).

In addition, we excluded that PC cell surface translocation was coupled to PC synthesis. First, [^{14}C]PC synthesis from [^{14}C]palmitoylcarnitine was increased in energy-depleted erythrocytes, whereas [^{14}C]PC cell surface translocation was reduced. Second, [^{14}C]PC translocation could be restored in energy-depleted erythrocytes by incorporation of ATP without inducing PC synthesis. Third, in a pulse-chase approach we were able to exchange a total amount of [^{14}C]PC from the cell surface that exceeded steady state cell surface [^{14}C]PC, demonstrating ongoing [^{14}C]PC translocation in the absence of further [^{14}C]PC labeling during the chase. Because lateral lipid diffusion in membranes is roughly 1 $\mu m^2/s$ (59) with an average cell diameter of 10 μm, it is difficult to understand how labeled PC could form a lipid pool that does not mix with bulk PC. We therefore conclude that the cell surface exposure of labeled PC reflects the active outward translocation of bulk PC.

In view of a rapid cell surface translocation of mass PC we would expect a compensatory PC inward flux to maintain the steady state plasma membrane PC distribution. When labeling the erythrocyte surface for 2 h with [^{14}C]arachidonoyl-PC at 37 °C only 65% of the labeled PC was accessible to PLA_2, whereas this was 92% when the cells were labeled at 4 °C. We concluded that after 2 h at 37 °C, [^{14}C]arachidonoyl-PC had translocated to the inside and had almost reached its steady state distribution (61% at the surface). If PC plasma membrane asymmetry was maintained by the dynamic equilibration of PC inward and outward fluxes, a half-time of 30 min for PC outward translocation (1-[^{14}C]palmitoyl-PC) would suggest a half-time of inward equilibration in the range of 1–2 h. Such a time frame of apparent PC inward translocation cannot be excluded from our results on [^{14}C]arachidonoyl-PC.

However, data in the literature showed a slow PC inward equilibration that positively depended upon the degree of PC unsaturation. 1-Palmitoyl-2-linoleoyl-PC, 1-palmitoyl-2-arachidonoyl-PC, and dipalmitoyl-PC exhibited half-times of inward equilibration of 2.9, 9.7, and 26.3 h, respectively (8). These PC species account for 26.9, 5.1, and 4.6% of total human erythrocyte PC (60), suggesting that the half-time of 1-palmitoyl-2-linoleoyl-PC approaches best the apparent inward translocation of total PC. Discrepancies in the determined time scale of apparent PC inward translocation in this study and those in the literature may be the result of differences in the cell surface assays. In this study, a 5-min PLA_2 incubation degraded 92% of PC that was inserted into the outer bilayer leaflet and 61% of total PC. However, when PLA_2 was used in combination with sphingomyelinase for 2 h, 76% of total PC was degraded (1, 2, 8). Sphingomyelinase can induce lipid translocation (61) and may render part of the inner leaflet PC available for degradation. If so, the 2-h PLA_2 and sphingomyelinase method underestimates the rate of PC inward translocation.

Natural PC Cell Surface Translocation and the Putative Role of ABC Transporters—ABC transporters have been widely associated with the outward translocation of lipids. Abcb4-mediated PC translocation is indispensable for PC excretion into bile (17). However, Abcb4 expression is mainly restricted to liver (62). The efflux of cellular PC toward ApoA-1 was associated with the broadly expressed ABCA1 and with ABCA7 (63, 64) and translocation of short chain lipid analogs was demonstrated for ABCB1 and ABCC1 (19, 23, 56, 58). Inhibitors of these transporters reduced C_6-NBD-PC outward translocation by an endogenous translocase in erythrocytes (23, 46) and fibroblasts.[2]

The inhibition of natural PC cell surface translocation in erythrocytes and fibroblasts by glibenclamide and vanadate, both general inhibitors of ABC transporters that affect ATP at the nucleotide-binding folds (65–67), could indicate that active translocation of endogenous PC in non-hepatic cells is also mediated by ABC transporters. In contrast to C_6-NBD-PC, natural PC translocation in human erythrocytes was insensitive toward the more specific ABC transporter inhibitors PSC833 and MK571, suggesting that ABCB1 and ABCC1 are involved in C_6-NBD-PC but not in natural PC translocation. Conversely, in erythrocytes from mice with homozygous disruption of the *abcb1a/1b* plus *abcc1*, *abcb1a/1b*, or *abcb4* genes, natural [^{14}C]PC cell surface translocation was reduced, supporting a role of Abcb1a/1b and Abcb4 in natural PC translocation. This discrepancy between the inhibitory profile of [^{14}C]PC outward translocation and results on knockout cells might be because of differences between human and mouse erythrocytes, like the absence of Abcb4 from human, but presence in mouse erythrocytes (see Ref. 68). Alternatively, the competitive inhibitor PSC833 could affect translocation of C_6-NBD-PC but not natural PC as was also seen after oral administration of PSC833 in mice, which inhibited Abcb4-mediated biliary excretion of drugs but not PC (69).

Besides Abcb1a/1b and Abcb4, other glibenclamide- and vanadate-sensitive translocators, such as proteins of the ABCA subfamily, could also be involved in outward PC translocation. Natural PC could be the substrate for more than one translocator as was seen for short chain NBD-PC (ABCB1 and C1) (19, 23) and for the inward translocation of natural PE and NBD-PC, -PE, and -PS (4). Final proof requires the direct demonstration of PC translocation by the purified protein in chemically defined proteoliposomes.

Physiological Implications of Plasma Membrane PC Cell Surface Translocation—We found that translocation of newly synthesized PC to the cell surface of fibroblasts was not affected in the absence of vesicle trafficking, demonstrating that bulk PC reaches the plasma membrane by a non-vesicular pathway in accordance with Kaplan and Simoni (52) but also that PC efficiently translocates from the inside to the outside of the plasma membrane. Cell surface lipid translocation is mostly associated with the exposure of PS and its signaling functions, *e.g.* in apoptosis. However, the active outward translocation of mass PC is probably not involved in lipid signaling, but rather affects plasma membrane dynamics. Vesicle fusion and fission were suggested to cause some lipid scrambling (70). With the total plasma membrane recycling in tens of minutes (71), the maintenance of lipid asymmetry would require massive inward and outward lipid translocation. Lipid translocation was also suggested to induce cellular shape changes (72, 73) that occur during cell movement, and to play an active role in vesicle budding (4, 6, 74). In living cells, rapid translocation of exogenous aminophospholipids toward the inside of the plasma membrane enhanced endocytosis, whereas lipids remaining at the cell surface inhibited the process (6). Similarly, outward translocation of bulk PC could counteract the aminophospholipid inward translocation, and reduced lipid outward translocation should then increase endocytosis. Strikingly, glibenclamide concentrations that inhibited PC cell surface translocation in the present study enhanced endocytosis in

[2] N. Kälin and G. van Meer, unpublished observations.

fibroblasts (75). Presently, we have no information concerning the relative importance of in- and outward phospholipid translocation for membrane trafficking in nucleated cells.

It is tempting to speculate that PC translocation, PC synthesis, and vesicle trafficking are part of a coordinated network that regulates the dynamics of cellular membranes. The identification and characterization of the PC translocator(s) will shed further light upon the mechanisms involved in plasma membrane lipid asymmetry and may be important for unraveling the basic mechanisms of membrane trafficking.

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