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Synthesis and Properties of Radioiodinated Phospholipid Analogues That Spontaneously Undergo Vesicle–Vesicle and Vesicle–Cell Transfer†

Alan J. Schroit* and John W. Madsen

ABSTRACT: An efficient method for the synthesis and purification of a variety of iodinated phospholipid analogues is described. 1-Acyl-2-[[[3-(3-[^{125}I]iodo-4-hydroxyphenyl)propionyl]amino]caproyl]phosphatidylcholine (^{125}I-PC) was prepared by alkylation of 1-acyl-2-(aminocaproyl)phosphatidylcholine with monoiodinated Bolton–Hunter reagent. ^{125}I-Labeled phosphatidic acid, phosphatidylethanolamine, and phosphatidylserine were produced from ^{125}I-PC by phospholipase D catalyzed base exchange in the presence of ethanolamine or L-serine. All of these lipid analogues transferred readily from donor vesicles into recipient membranes. When an excess of acceptor vesicles was mixed with a population of donor vesicles containing the iodinated analogues, approximately 50% of the ^{125}I-labeled lipids transferred to the acceptor vesicle population. In addition, under appropriate incubation conditions, these lipids were observed to transfer from vesicles to mammalian cells. Autoradiographic analysis of ^{125}I-labeled lipids extracted from the cells after incubation with vesicles at 2 °C for 60 min revealed that a large proportion of the ^{125}I-labeled phosphatidic acid was metabolized to ^{125}I-labeled diglyceride and ^{125}I-labeled phosphatidylcholine, whereas no metabolism of exogenously supplied ^{125}I-labeled phosphatidylethanolamine or ^{125}I-labeled phosphatidylcholine could be detected.

Several biochemical and biological studies have demonstrated that certain phospholipid molecules can exchange/transfer between populations of lipid vesicles and between vesicles and cells. Although this phenomenon has been shown to occur with unmodified phospholipids (Pagano & Huang, 1975; Papahadjopoulos et al., 1976; Martin & MacDonald, 1976; Duckwitz-Peterlein et al., 1977; Kremer et al., 1977a; Sandra & Pagano, 1979; De Cuyper et al., 1980), additional insight into the mechanism involved in this process has been obtained through the use of fluorescent lipid analogues (Monti et al., 1977; Roseman & Thompson, 1980).

The elegant studies of Pagano and co-workers using a variety of vesicle–vesicle (Pagano et al., 1981a) and vesicle–cell (Struck & Pagano, 1980; Pagano et al., 1982) systems have demonstrated that certain acyl chain labeled phospholipid analogues can readily transfer from synthetic phospholipid vesicles. This has been shown to occur with 1-acyl-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]caproyl]phosphatidylcholine (NBD-PC),[1] NBD-PE, and NBD-PA but not with NBD-ceramide or NBD-PG, suggesting some dependence of the phenomenon on the composition of the polar head group. Indeed, various polar head group labeled phospholipid analogues of phosphatidylethanolamine such as *N*-NBD-PE, *N*-Rh-PE, *N*-fluoresceinyl-PE (Struck & Pagano, 1980), and ^{125}I-phenylpropionyl-PE (Schroit, 1982) have been shown to be nontransferable.

Although the molecular arrangements responsible for the transfer phenomenon are not completely defined, the solubility of the lipid monomer in water is apparently a factor (Roseman & Thompson, 1980; Nichols & Pagano, 1981). Thus, lipid tranfer occurs with a variety of lyso- (Weltzien, 1979), short-chain (Martin & MacDonald, 1976; De Cuyper et al., 1980; Tanaka et al., 1980, 1983), and unsaturated phospholipids (Pagano & Huang, 1975; Duckwitz-Peterlein et al., 1977; Sandra & Pagano, 1979) and also probably results from the attachment of relatively bulky aromatic groups to phospholipid fatty acyl side chains, as in the instance of some of the fluorescent analogues (Monti et al., 1977; Roseman & Thompson, 1980).

If this is indeed the case, then one might assume that the attachment of other aromatic moieties to the 2-position fatty acid of phospholipids might similarly affect and ultimately

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[1] Abbreviations: DOPC, dioleoylphosphatidylcholine; NBD-PC, 1-acyl-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]caproyl]phosphatidylcholine; *N*-NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; *N*-Rh-PE, *N*-(lissamine rhodamine B sulfonyl)-dioleoylphosphatidylethanolamine; Boc-ON, 2-[[(*tert*-butoxycarbonyl)-oxy]imino]-2-phenylacetonitrile; ^{125}I-BHR, succinimido 3-(3-[^{125}I]iodo-4-hydroxyphenyl)propionate, monoiodinated Bolton–Hunter reagent; PBS, Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline, pH 7.2; NH_2-PC, 1-acyl-2-(aminocaproyl)phosphatidylcholine; ^{125}I-PC, 1-acyl-2-[[[3-(3-[^{125}I]iodo-4-hydroxyphenyl)propionyl]amino]caproyl]phosphatidylcholine; ^{125}I-PA, 1-acyl-2-[[[3-(3-[^{125}I]iodo-4-hydroxyphenyl)-propionyl]amino]caproyl]phosphatidic acid; ^{125}I-PE, 1-acyl-2-[[[3-(3-[^{125}I]iodo-4-hydroxyphenyl)propionyl]amino]caproyl]phosphatidylethanolamine; ^{125}I-PS, 1-acyl-2-[[[3-(3-[^{125}I]iodo-4-hydroxyphenyl)-propionyl]amino]caproyl]phosphatidylserine; LUV, large unilamellar vesicle(s); SUV, small unilamellar vesicle(s).

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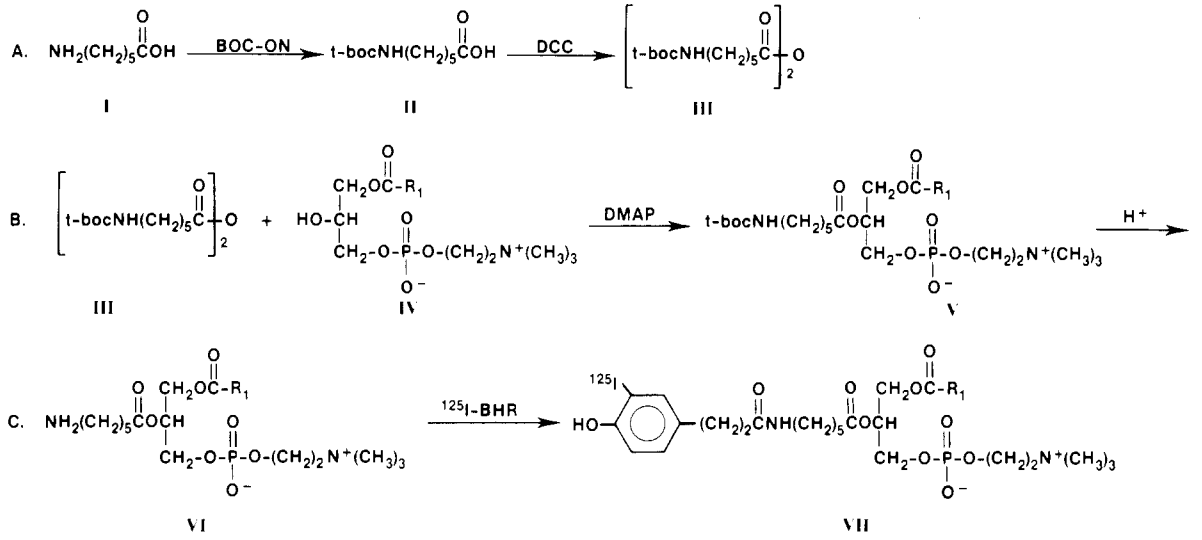

FIGURE 1: Synthesis of ^{125}I-PC. (A) Preparation of blocked fatty acid anhydride. (B) Acylation and deblocking of lysophosphatidylcholine. (C) Acylation of NH_2-PC with ^{125}I-BHR.

facilitate lipid transfer. In line with these observations, we describe here the synthesis, purification, and uses of several phospholipid analogues labeled with ^{125}I at the acyl chain. These compounds are of high specific activity and readily undergo vesicle–vesicle and vesicle–cell transfer. In addition, these analogues should be ideal exogenous substrates for the study of lipid metabolic pathways in mammalian cells.

Experimental Procedures

Materials. Dioleoylphosphatidylethanolamine, dipalmitoylphosphatidylethanolamine, lysophosphatidylcholine, DOPC, *N*-NBD-PE, and NBD-PC were purchased from Avanti Polar Lipids (Birmingham, AL). Boc-ON was obtained from Pierce Chemical Co. (Rockford, IL). *N*-Rh-PE was prepared by reacting lissamine rhodamine B sulfonyl chloride (Molecular Probes, Junction City, OR) with dioleoylphosphatidylethanolamine, as previously described (Struck et al., 1981). ^{125}I-BHR (sp act. ~2000 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Phospholipase A_2 (hog pancreas) and phospholipase D (cabbage) were products of Boehringer Mannheim. Phospholipase C (*Clostridium perfringens*) was obtained from Calbiochem. Thin-layer chromatography was carried out by using activated silica gel 60 thin-layer plates (Merck) in $CHCl_3$/MeOH/H_2O (65:25:4) unless specifically stated otherwise. The presence of ^{125}I-containing compounds was assessed by autoradiography using Kodak XAR-5 film. Radiation was measured with an Amersham/Searle Model 1185 automatic γ counter. Fluorescence was quantified with a Farrand MK II spectrophotofluorometer.

Cells. Monolayer cultures of the UV radiation induced UV-2237 fibrosarcoma, which is syngeneic to C3H mice, were grown to confluence in Eagle's minimum essential medium supplemented with L-glutamine and 10% fetal bovine serum at 37 °C in a humidified atmosphere of 5% CO_2 in air. Cell suspensions were prepared by brief trypsinization at 22 °C. The single cell suspension was then extensively washed with PBS and kept on ice until used.

Synthesis of 1-Acyl-2-(aminocaproyl)phosphatidylcholine (NH_2-PC). Figure 1 shows the multistep procedure for the preparation of NH_2-PC (VI). The reactive amino group of aminocaproic acid (I) was protected by *tert*-butoxycarbonylation (II), reacted with dicyclohexylcarbodiimide to yield the [(*t*-Boc)amino]caproic anhydride (III), condensed with lysophosphatidylcholine (IV) to yield [[(*t*-Boc)amino]-caproyl]phosphatidylcholine (V), and finally deblocked with anhydrous HCl (VI).

tert-Butoxycarbonylation of aminocaproic acid was carried out as described by W. Shaw (personal communication). Aminocaproic acid was dissolved in H_2O/MeOH/tetrahydrofuran (3:1:3) and adjusted to pH 10 with triethylamine. Solid Boc-ON (10% mole excess) was added, and the solution was mixed overnight at room temperature. The organic solvents were then removed under reduced pressure and extracted with $CHCl_3$/MeOH/0.1 N HCl (2:2:1.8). The lipid phase was removed, evaporated under reduced pressure followed by high vacuum, and finally suspended in dry benzene. Analysis of the product, [*N*-(*tert*-butoxycarbonyl)amino]-caproic acid, by thin-layer chromatography revealed a single spot (R_f = 0.71) which was very weakly ninhydrin positive and visible only following prolonged heating [R_f[NH_2-$(CH_2)_5$COOH] = 0.1].

[(*t*-Boc)amino]caproic anhydride was made by reacting 1.6 mmol of the blocked acid with 0.8 mmol of dicyclohexylcarbodiimide in 2 mL of dry benzene for 2 h at room temperature (Selinger & Lapidot, 1966). The dicyclohexylurea formed was removed by filtration, and the clear solution was dried under a stream of nitrogen. The residue was used without further purification.

The procedure for acylation of lysophosphatidylcholine with the blocked fatty acid anhydride was slightly modified from that described by Gupta et al. (1977). Briefly, 0.2 mmol of lysophosphatidylcholine in 5 mL of $CHCl_3$/pyridine (4:1) containing 50 mg of *N*,*N*-dimethyl-4-aminopyridine was added to the dry [(*t*-Boc)amino]caproic anhydride. The reaction vessel was flushed with nitrogen and mixed at 50 °C for 24 h. The solvents were then removed under reduced pressure, and the residue was dissolved in 5 mL of $CHCl_3$/MeOH/H_2O (4:5:1) and slowly chromatographed through a 2 × 30 cm column of Dowex 50W-X8 resin that had been washed with the same solvent. The catalyst-free product was then partitioned by adding $CHCl_3$ and 0.1 N HCl (1:4 v/v). The organic phase was taken to dryness, dissolved in $CHCl_3$, and applied to a 23 × 1.5 cm column of activated prewashed silica gel. The column was washed with 100 mL of $CHCl_3$, followed by 100-mL aliquots of $CHCl_3$ containing increasing amounts of MeOH. Analysis of the product which eluted with $CHCl_3$/MeOH (7:5) by thin-layer chromatography revealed

a single phosphate-positive and weakly ninhydrin-positive spot visible only after prolonged heating (R_f = 0.21). The final yield of pure [[(*t*-Boc)amino]caproyl]phosphatidylcholine with respect to the initial amount of lysophosphatidylcholine used was ~30%.

[[(*t*-Boc)amino]caproyl]phosphatidylcholine was deblocked with anhydrous HCl as previously described (Nichols & Pagano, 1981). The reaction mixture was extracted, and the lipid phase was purified by preparative thin-layer chromatography. The product, NH_2-PC (R_f = 0.02), was recovered from the plate by extraction and was strongly ninhydrin and phosphate positive.

Preparation of ^{125}*I-PC.* ^{125}I-PC was synthesized by reacting ^{125}I-BHR (1 mCi = ~4.5 × 10^{-10} mol) (Rudinger & Ruegg, 1973; Bolton & Hunter, 1973) with 100 μg of NH_2-PC in 200 μL of $CHCl_3$/MeOH (1:2) containing 1 μL of triethylamine for 18 h at 0 °C. Five milliliters of $CHCl_3$/MeOH/H_2O (2:2:1.8) was then added, and the mixture was vigorously vortexed. After centrifugation, the lower organic phase was removed to a clean tube and washed with water; the ^{125}I-PC was purified by preparative thin-layer chromatography on activated silica gel thin-layer plates (R_f = 0.17) and recovered as previously described (Schroit, 1982). Typical yields of pure ^{125}I-PC were in the range of 45–55% with respect to the initial amount of ^{125}I-BHR employed.

Preparation of ^{125}*I-PA,* ^{125}*I-PE, and* ^{125}*I-PS.* ^{125}I-PE and ^{125}I-PS were prepared from ^{125}I-PC by phopholipase D catalyzed base exchange in the presence of ethanolamine or L-serine, respectively (Comfurius & Zwaal, 1977). ^{125}I-PA was isolated as a byproduct from the ^{125}I-PC/ethanolamine reaction mixture. All of the derivatives were purified by thin-layer chromatography in $CHCl_3$/MeOH/H_2O (65:25:4) except ^{125}I-PS, which required two-dimensional chromatography [first dimension, $CHCl_3$/MeOH/28% NH_4OH (65:35:5); second dimension, $CHCl_3$/MeOH/acetic acid/acetone/H_2O (5:1:1:2:0.5)], and they were eluted from the plates following identification by autoradiography. The amino-containing phospholipids were positively identified on the basis of their reactivity with picryl sulfonic acid as previously described (Schroit & Pagano, 1981) by assessing their concomitant altered mobility on thin-layer chromatographic plates.

Lipid Vesicles. Unilamellar vesicles of various sizes were prepared by ethanol injection (Kremer et al., 1977b) as previously described. Briefly, LUV were made by drying 10 mg of total lipid (DOPC with trace amounts of ^{125}I-labeled phospholipid analogue, *N*-NBD-PE, NBD-PC, and/or *N*-Rh-PE) under a stream of dry nitrogen gas and further dried under high vacuum. The lipids were then dissolved in 250 μL of absolute ethanol and injected with stirring into 3.2 mL of PBS. The resulting vesicle preparation was subsequently dialyzed overnight against several liters of the same buffer. SUV were prepared by the same general procedure except that a total of 0.5 mg of lipid in 167 μL of ethanol was injected into 2.2 mL of PBS. In some experiments, "contaminating" LUV or multilamellar vesicles were removed by passing the preparations over a Bio-Gel A-15m column and by collecting only the included vesicle peak.

Vesicle–Vesicle Exchange. Vesicle–vesicle exchange experiments were carried out essentially as described by Pagano et al. (1981a) except that the extent of lipid transfer was assessed by using Bio-Gel A-15m column chromatography. Briefly, fluorescent or radiolabeled donor SUV and acceptor LUV were prepared as described above. Donor and acceptor vesicles were then mixed at a 1:6 ratio (w/w), and the mixture was incubated for 5 min at room temperature. The mixed vesicle population (250 μL) was then separated according to size on a Bio-Gel A-15m column (49 × 0.9 cm) precalibrated with known standard vesicle preparations. Fractions were collected (~0.8 mL), and the presence of vesicles in the individual fractions was assessed by a combination of light-scatter, fluorescence, and radioactivity determinations. The extent to which the ^{125}I-labeled lipids exchanged between the various vesicle preparations was assessed by calculating the standard normal distribution of radiation in the SUV donor and LUV acceptor populations by using nonexchangeable fluorescent lipids (*N*-Rh-PE and *N*-NBD-PE) as population markers.

Vesicle–Cell Incubation. Washed UV-2237 cells (10^7) were suspended in 1 mL of the indicated SUV preparations (200 μg of lipid/mL) and incubated for 1 h at 2 °C. The cells were then washed 4 times with cold PBS to remove nonadherent vesicles and were transferred to a new tube following each wash. The amount of lipid transferred to the cells was determined by extracting the washed cells (suspended in several drops of PBS) with 3.8 mL of $CHCl_3$/MeOH/0.1 N HCl (1:2:0.8) for 1 h at 0 °C. Insoluble material was removed by centrifugation, and the solvent was partitioned by adding 1 mL of $CHCl_3$ and 1 mL of water, followed by vigorous vortexing and centrifugation. The organic phase was removed and dried under a stream of nitrogen. The lipid residue was then dissolved in 1 mL of ethanol. The ratios of radiolabeled to fluorescent lipids in the extracts (as determined by scintillation and fluorescent spectrometry, respectively) were compared to that of the initial vesicle population, which was extracted in an identical manner. The amount of NBD- and rhodamine-labeled lipids present in these extracts was determined by using standard curves generated from known amounts of these analogues.

Fluorescence Measurements. The relative fluorescence of vesicles or vesicle-treated cells was determined in 1-mL samples at 22 °C. By use of crossed polarizers and 5-nm band-pass slits to minimize light scatter, the amount of NBD-PC, *N*-NBD-PE, and *N*-Rh-PE in the intact vesicles or various extracts was assessed by direct excitation at 470 (λ_{em} 525 nm) and 560 nm (λ_{em} 590 nm), respectively. The fluorescence of vesicles containing self-quenching concentrations of NBD-PC or *N*-NBD-PE (Schroit & Pagano, 1981) and of vesicles containing both the NBD and rhodamine analogues was quantified only in extracted or lysed samples (1% Triton X-100), which abrogates all energy-transfer effects (Pagano et al., 1981a).

Results

Sensitivity of ^{125}*I-PC to Phospholipases.* In order to verify the labeling specificity of ^{125}I-BHR with NH_2-PC, we subjected the purified product to hydrolysis with phospholipase A_2 and phospholipase C as described by Kates (1972). Both reactions resulted in complete conversion of the compound to the corresponding ^{125}I-labeled aminocaproic acid and ^{125}I-labeled 1-acyl-2-(aminocaproyl)diglyceride, respectively (Figure 2). In addition, only minor amounts of radiation (~0.15%) could be detected in the water-soluble phosphorylcholine-containing fraction obtained by phospholipase C hydrolysis.

^{125}I-PA, ^{125}I-PE, and ^{125}I-PS were produced by phospholipase D catalyzed base exchange of purified ^{125}I-PC (Figure 3). In agreement with previous investigations, all of the phosphatidylcholine was converted into the respective phospholipid derivatives, although the major byproduct was diglyceride and not phosphatidic acid (Table I). The mechanism responsible for diglyceride formation is not known, although attachment of the relatively bulky *N*-acyl group to the

Table I: Products Obtained by Phospholipase D Catalyzed Base Exchange of ^{125}I-PC

base	lipid yield (%)[a]					
	^{125}I-PC	^{125}I-PA	^{125}I-PE	^{125}I-PS	^{125}I-labeled diglyceride	uniden-tified
ethanolamine	<1	21	34	0	41	3
serine	<1	4	0	3	88	4

[a] Yields based on the total amount of radiation recovered from the thin-layer chromatography plates from one representative experiment.

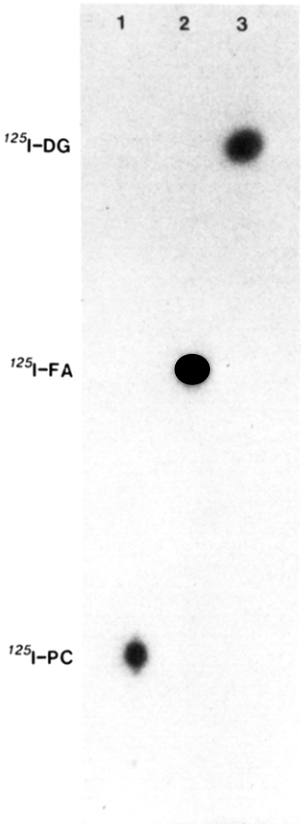

FIGURE 2: Thin-layer autoradiograph of ^{125}I-PC (left lane) hydrolyzed with phospholipase A_2 (middle lane) and phospholipase C (right lane) in $CHCl_3$/MeOH/H_2O (65:25:4). Abbreviations: ^{125}I-DG, ^{125}I-labeled diglyceride; ^{125}I-FA, ^{125}I-labeled fatty acid.

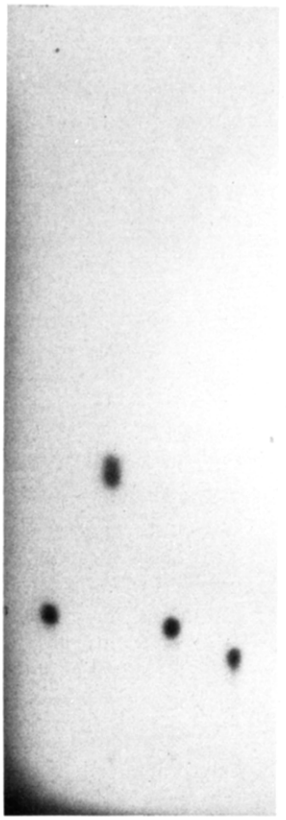

FIGURE 3: Thin-layer autoradiograph of purified (from left to right) ^{125}I-PC, ^{125}I-PE, ^{125}I-PA, and ^{125}I-PS chromatographed in $CHCl_3$/MeOH/H_2O (65:25:4).

fatty acid probably results in altered enzyme specificity. Indeed, the extent of diglyceride formation was independent of the batch and source of phospholipase D, and the inclusion of milligram amounts of DOPC in the reaction mixture did not result in the formation of diglyceride derived from the DOPC.

Pure ^{125}I-PE and ^{125}I-PA were obtained in relatively good yields (34% and 21%, respectively) following purification by thin-layer chromatography, whereas the yield of ^{125}I-PS was poor (~3%) and two-dimensional chromatography was required to separate it from the ^{125}I-PA, which appeared concomitantly. Positive identification of ^{125}I-PE (R_f = 0.4) and ^{125}I-PS (R_f = 0.14) was accomplished by derivatization with picryl sulfonic acid, which resulted in their complete conversion to faster migrating trinitrophenyl derivatives.

Exchange of ^{125}I-Labeled Phospholipid Analogues between Vesicles. When a population of donor vesicles containing ^{125}I-PC was mixed with a population of acceptor vesicles, approximately 50% of the ^{125}I-labeled lipid transferred from the donor to the acceptor population (Figure 4 and Table II). In contrast, the nonexchangeable LUV or SUV marker lipids (*N*-NBD-PE or *N*-Rh-PE, respectively) did not transfer between the two vesicle populations. The extent of ^{125}I-PA and ^{125}I-PE transfer (Table II) was similar to that of ^{125}I-PC, suggesting that the phospholipid polar head group of these particular lipids has only minimal effects on the exchangeable nature of these analogues.

Table II: Transfer of ^{125}I-Labeled Phospholipids between SUV and LUV Populations[a]

lipid in SUV donors	no. of incubations with LUV acceptors[b]	distribution of lipid (cpm × 10^{-3})[c]		% lipid exchanged
		SUV	LUV	
^{125}I-PC	1	82.6	94.0	53.2
	2	5.9	0.3	5.2
^{125}I-PE	1	91.6	90.7	49.8
	2	6.5	0.3	4.2
^{125}I-PA	1	128.3	116.8	47.7
	2	10.3	0.9	7.7

[a] SUV containing ^{125}I-labeled phospholipids were prepared as described and incubated for 5 min at room temperature with a 6-fold excess (w/w) of acceptor LUV. The LUV and SUV populations were then separated by chromatography on Bio-Gel A-15m. The extent of ^{125}I-labeled lipid transfer between the two vesicle populations was determined by the amount of lipid in each of the populations as determined by the presence of *N*-NBD-PE and *N*-Rh-PE (see Experimental Procedures). [b] For the second exchange, SUV recovered from the Bio-Gel A-15m column were reincubated with fresh LUV acceptor vesicles and rechromatographed. [c] Representative experiment of 10 individual runs. Variations among individual experiments were <10%.

Table III: Cell Uptake of ^{125}I-Labeled Phospholipid Analogues[a]

^{125}I-labeled phospholipid analogue	initial vesicle suspension		lipid uptake/10^7 cells		^{125}I-labeled lipid (cpm × 10^{-3})[c]/ng of *N*-Rh-PE	
	^{125}I-labeled lipid (cpm × 10^{-3})	*N*-Rh-PE (ng)	^{125}I-labeled lipid (cpm × 10^{-3})	*N*-Rh-PE[b] (ng)	vesicles	cells
^{125}I-PC	9138	5216	105	17	1.75	6.18
^{125}I-PE	8349	4964	74	8	1.68	9.25
^{125}I-PA	8674	4442	188	11	1.95	17.09
^{125}I-PS	447	1340	14	5	0.33	2.80

[a] 10^7 cells were incubated with DOPC SUV (200 μg of lipid/mL) containing 3% *N*-Rh-PE (w/w) and ~4.0 μCi of ^{125}I-labeled phospholipids for 60 min at 2 °C. The fraction of cell-associated fluorescence and radiation was determined after extensive washings. [b] The amount of lipid uptake by the cells was calculated from standard curves generated from known amounts of fluorescent lipid. [c] Representative results of three individual experiments. Variations in the final ratios of ^{125}I-labeled lipid/*N*-Rh-PE were <10%.

Table IV: Cell Uptake of ^{125}I-PC and NBD-PC[a]

lipid uptake/10^7 cells[b]			^{125}I-PC (cpm × 10^{-3})/ng of *N*-Rh-PE		^{125}I-PC (cpm × 10^{-3})/ng of NBD-PC	
^{125}I-PC (cpm × 10^{-3})	*N*-Rh-PE[c] (ng)	NBD-PC[c] (ng)	vesicles	cells	vesicles	cells
148	15	468	1.47	9.87	0.44	0.32

[a] SUV were composed of DOPC containing 10% (w/w) NBD-PC, 3% (w/w) *N*-Rh-PE, and ~4.0 μCi of ^{125}I-PC. [b] Incubation conditions as described in Table III. [c] The amount of lipid uptake by the cells was calculated from standard curves generated from known amounts of fluorescent lipid.

For determination of the maximum amount of transferable ^{125}I-labeled phospholipid, donor SUV were subjected to an additional incubation with acceptor LUV following their initial purification by gel filtration. As can be seen from the data presented in Table II, only ~5% of the total ^{125}I-labeled phospholipids present in the preexchanged donor population were observed to transfer, suggesting that most of these phospholipids were removed from the external leaflet of the vesicle bilayer and that the remaining phospholipids were confined to the inner leaflet.

Transfer of ^{125}I-Labeled Phospholipids from Vesicles to Cells. When SUV composed of DOPC, nonexchangeable *N*-Rh-PE, and ^{125}I-PC, ^{125}I-PE, ^{125}I-PA, or ^{125}I-PS were incubated with UV-2237 fibrosarcoma cells at 2 °C, only the iodinated analogues were transferred to the cells in significant amounts (Table III). Since *N*-Rh-PE does not transfer from vesicles to either acceptor vesicle populations or cells (Struck & Pagano, 1980; Pagano et al., 1981a), these results strongly suggest that uptake of the ^{125}I-labeled phospholipid analogues by the cells was due primarily to lipid transfer (or possibly exchange) and only minimally to the binding of intact vesicles to cells. Indeed, in every instance, the ratio of radiation to rhodamine fluorescence in the vesicle-treated cells was substantially greater than that in the initial vesicle population. In addition, from the data presented in Table III, it can be seen that <150 molecules of ^{125}I-labeled lipid/cell can be easily detected. On the basis of the degree of enhanced cell-associated radiation/fluorescence in relation to that of the applied vesicle suspension, the potential for lipid transfer appears greatest for ^{125}I-PA ≅ ^{125}I-PS > ^{125}I-PE > ^{125}I-PC.

For comparison of the relative transferability of ^{125}I-PC with the known rapid transfer of NBD-PC (Struck & Pagano, 1980; Nichols & Pagano, 1981), cells were incubated with vesicles containing both ^{125}I-PC and NBD-PC. As shown in Table IV, the ratio of cell-associated ^{125}I-PC to NBD-PC was similar to that of the applied vesicle suspension, suggesting a similar degree of lipid transfer for both analogues, whereas again only limited amounts of *N*-Rh-PE became cell associated.

Analysis of the ^{125}I-labeled phospholipids extracted from cells treated at 2 °C with the various vesicle preparations containing the labeled lipids is shown in Figure 5 and Table V. No metabolism of ^{125}I-PC or ^{125}I-PE could be detected.

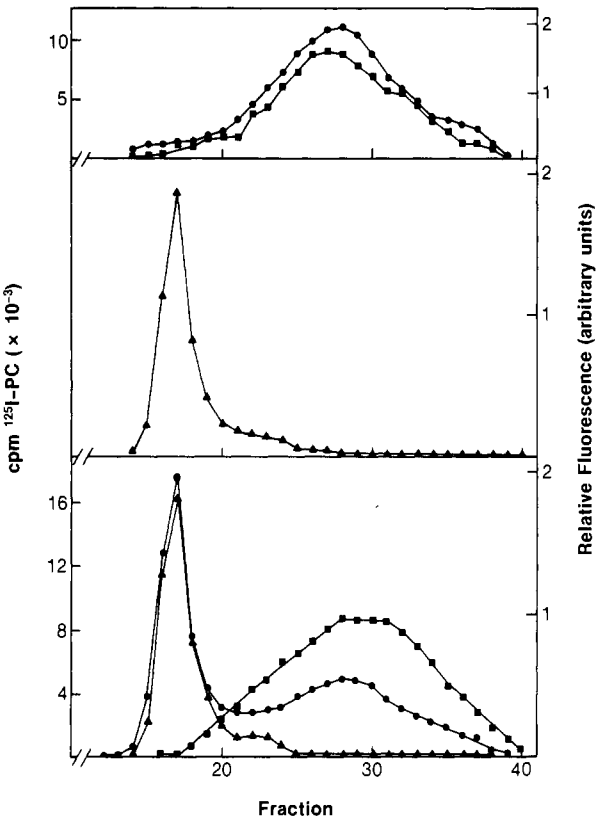

FIGURE 4: Vesicle–vesicle transfer of ^{125}I-PC between small (SUV) and large (LUV) vesicle populations. SUV were formed from DOPC/*N*-Rh-PE/^{125}I-PC (99:1:trace) and LUV from DOPC/*N*-NBD-PE (99:1). Bio-Gel A-15m chromatography of SUV alone (top), LUV alone (middle), and a mixture of LUV and SUV (6:1 w/w) after a 5-min incubation at 20 °C (bottom). (●) ^{125}I-PC; (■) *N*-Rh-PE; (▲) *N*-NBD-PE.

However, when the cells were incubated with vesicles that contained ^{125}I-PA, approximately 80% of the lipid was metabolized to ^{125}I-PC and ^{125}I-labeled diglyceride (Table V). The observed metabolism at 2 °C was confirmed to be enzymatic in nature, in that boiling of the cells for 10 min prior to the addition of ^{125}I-PA completely abrogated its conversion into other iodinated lipid analogues. In addition, ^{125}I-PA

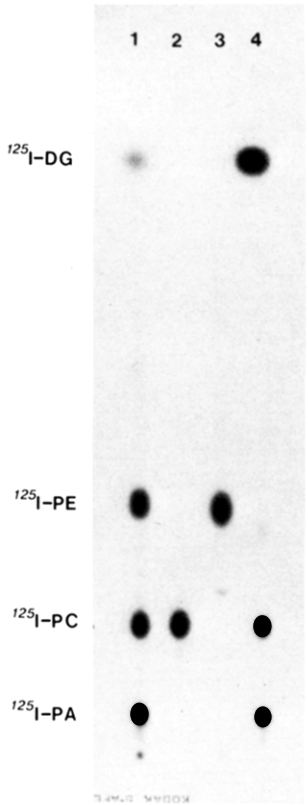

FIGURE 5: Thin-layer autoradiograph of extracts from cells treated with vesicles containing ^{125}I-labeled phospholipids. Incubation conditions were as described in Table III. Lane 1, ^{125}I-labeled lipid standards; lanes 2, 3, and 4, lipid extracts from cells treated with DOPC SUV containing ^{125}I-PC, ^{125}I-PE, and ^{125}I-PA, respectively, chromatographed in $CHCl_3$/MeOH/28% NH_4OH (65:35:5). 2000 cpm (0.2×10^6 cell equivalents) spotted.

Table V: Analysis of ^{125}I-Labeled Lipids in ^{125}I-PA-Treated Cells[a]

	^{125}I-labeled lipids (%)[b]	
	expt I	expt II
^{125}I-PA	22.1	13.8
^{125}I-PC	23.9	15.6
^{125}I-DG	55.2	69.8
unidentified	1.5	0.8

[a] Incubation conditions as described in Table III. [b] Extracted lipids were separated by thin-layer chromatography. The autoradiographically identified products were quantified by scraping and counting appropriate areas of the plate.

included in neutral or acidic single-phase cell extraction mixtures remained intact (results not shown).

Discussion

In this report, we describe an efficient method for the synthesis of transferable/exchangeable iodinated phospholipid analogues. The technique involves protection of the reactive amino group of aminocaproic acid, followed by esterification of lysophosphatidylcholine, removal of the protecting group, and finally alkylation with commercially available ^{125}I-BHR. The product, ^{125}I-PC, is obtained in high yield (~50% maximum theoretical yield) and has extremely high specific activity (~2000 Ci/mmol). Through the use of phospholipase D catalyzed base exchange in the presence of appropriate bases, ^{125}I-PE, ^{125}I-PA, and ^{125}I-PS were also prepared.

Positive identification of ^{125}I-PC was obtained by hydrolysis of the compound with phospholipase A_2 and phospholipase C, which resulted in the complete conversion of the ^{125}I-PC to ^{125}I-labeled fatty acid and ^{125}I-labeled diglyceride. In addition, essentially no radiation could be detected in either the lysophosphatidylcholine or the phosphorylcholine produced by the lipases, suggesting that ^{125}I-BHR alkylation is highly specific for the reactive amino group of the 2-position fatty acid. Indeed, previous attempts to label phosphatidylcholine with ^{125}I-BHR failed (Schroit, 1982). Although conversion of ^{125}I-PC to the other phospholipid derivatives by phospholipase D was straightforward, relatively large amounts of ^{125}I-labeled diglyceride were formed. This is somewhat surprising in that phosphatidic acid is usually the major byproduct when phosphatidylcholines of natural structure are used as substrates. The reason for diglyceride formation is not known; however, phospholipases are highly stereospecific, and the attachment of the bulky phenylpropionyl group to the β-position fatty acid probably results in altered lipid stereochemistry.

All of the iodinated phospholipid derivatives produced were found to be stable for at least 3 months when stored at −70 °C. In addition, no degradation of ^{125}I-PC, ^{125}I-PA, or ^{125}I-PS could be detected following vesicle preparation by ultrasonication (using a probe-type sonifier, 2 °C for 10 min), whereas ^{125}I-PE was completely destroyed (unpublished results). Because of this, we routinely prepared vesicles by ethanol injection (Kremer et al., 1977b) whenever possible.

All of the ^{125}I-labeled phospholipid analogues tested were efficiently transferred to acceptor vesicle populations.[2] When SUV donor vesicles containing ^{125}I-labeled phospholipids were used, approximately 50% of the lipid in the donor population was transferred to the acceptor population. The extent of lipid transfer is similar to the extent of NBD-PC transfer from SUV (Pagano et al., 1981a). Finally, it should be noted that the appearance of ^{125}I-labeled lipid in the LUV acceptor population was not caused by vesicle–vesicle adhesion because the nonexchangeable analogues, *N*-NBD-PE and *N*-Rh-PE (Pagano et al., 1981a), did not shift from one vesicle population to the other, as determined by the Bio-Gel A-15m column.

Lipid vesicles are known to interact with cells by various mechanisms, notably by fusion, absorption, or endocytosis, and/or lipid transfer (Pagano & Weinstein, 1978; Pagano et al., 1981b; Poste, 1980). With regard to lipid transfer, it appears that individual lipid molecules can undergo this process by virtue of soluble lipid monomer diffusion (Roseman & Thompson, 1980; Nichols & Pagano, 1981). Indeed, this finding is reinforced by the observations on the transfer/exchange capacity of "more polar" lipids such as lyso- (Weltzien, 1979), short-chain (Martin & MacDonald, 1976; De Cuyper et al., 1980; Tanaka et al., 1980, 1983), and unsaturated phospholipids (Pagano & Huang, 1975; Duckwitz-Peterlein et al., 1977; Sandra & Pagano, 1979), which can transfer from vesicles into recipient membranes. Although the required lipid structure responsible for the transfer phenomenon has not been completely defined, the attachment of relatively bulky aromatic groups to phospholipid acyl side chains apparently enhances the potential for this process to occur. Thus, it has been shown that NBD-PC (Monti et al., 1977; Struck & Pagano, 1980), NBD-PE (Struck & Pagano, 1980), and NBD-PA (Pagano et al., 1982), which are labeled at the acyl chain, are efficiently transferred from vesicles to cells. In line with these observations, we have shown here that similarly ^{125}I-labeled phospholipid analogues are also efficiently transferred to cells, in contrast to lipids such as *N*-NBD-PE, *N*-Rh-PE (Struck &

[2] Due to the poor yield of ^{125}I-PS, only limited experiments were done with this compound.

Pagano, 1980), and ^{125}I-PE (Schroit, 1982), which are labeled at the polar head group and do not transfer.

The transfer of ^{125}I-PC, ^{125}I-PA, and ^{125}I-PE to the cells did not result in any readily apparent generalized toxicity effects. This was concluded from observations that the viability of vesicle-treated cells was identical with that of untreated cells (<95% viable cells determined by dye exclusion) and by the finding that plating efficiencies were unaltered (results not shown). Autoradiographic analysis of extracted cells treated at 2 °C with vesicles containing the iodinated phospholipid analogues revealed that only ^{125}I-PA was converted into other iodinated lipid products. Unlike ^{125}I-PC and ^{125}I-PE, incubation of the cells with vesicles containing ^{125}I-PA resulted in the production of significant amounts of ^{125}I-labeled diglyceride and ^{125}I-PC. These results are quite similar to the findings that NBD-PC is not metabolized, whereas NBD-PA is converted into other fluorescent products (Pagano et al., 1982) by V-79 fibroblasts at 2 °C. Whether these observations are directly representative of endogenous phosphatidic acid metabolism remains to clarified, although ^{125}I-PA does appear to be metabolized through pathways of known glycerolipid biosynthesis (Bell & Coleman, 1980). This suggests that those enzymes involved in these reactions do recognize ^{125}I-PA irrespective of the relatively bulky ^{125}I-labeled aromatic ring reporter group. We do, however, stress that studies using physical probes such as the analogues described herein reflect only those properties of the analogues itself, which may or may not be truly indicative of endogenous processes.

In summary, we have described a method for the preparation of several iodinated, readily transferable phospholipid analogues with physical properties similar to those of transferable fluorescent phospholipid analogues. They readily transfer into mammalian cells without concomitant mass vesicle absorption and/or fusion and, therefore, should be useful in studies of lipid metabolism. They should also permit detailed intracellular lipid localization studies by electron microscope autoradiography. Furthermore, since these lipids and their products are strong γ emitters which are unaffected by a variety of quenching phenomena commonly encountered with the use of other markers (i.e., fluorescence, ^{3}H, ^{14}C), minor lipid metabolites can be readily identified. Indeed, we were easily able to detect <1500 molecules of the ^{125}I-labeled lipids/cell.

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