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Novel radioactive phospholipid probes as a tool for measurement of phospholipid translocation across biomembranes

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In an attempt to develop a new method to measure transbilayer phospholipid translocation, with a higher sensitivity and higher temporal resolution, novel radioactive phospholipid probes (*C5-PC, *C5-PE, and *C5-PS) with a short acyl chain at the 2-position were synthesized. The *C5-PC probe was made by coupling lysophosphatidylcholine with [^{14}C]pentanoic acid, using *N,N*-carbonyldiimidazole as a coupling agent (yield 37%), and *C5-PE and *C5-PS were synthesized by exchanging the choline moiety of *C5-PC for ethanolamine and L-serine, respectively, as catalyzed by phospholipase D. The usefulness of the probes was confirmed by measuring phospholipid translocation across the human erythrocyte plasma membrane, in which the presence of aminophospholipid translocase was revealed using EPR techniques (Zachowski, A., Farve, E., Cribier, S., Herve, P. and Devaux, P.F. (1986) Biochemistry 25, 2585–2590). Using the present probes, ATP-dependent and SH-reagent-inhibitable translocation of *C5-PS and *C5-PE from outer to inner leaflets, which is characteristic to the translocation mediated by aminophospholipid translocase, was detected with a higher sensitivity than seen with the EPR technique. These radioactive phospholipid probes will be useful to measure phospholipid translocation with a high sensitivity and have the potential for application in measurements of transbilayer lipid-translocation for a wide variety of membranes.

Introduction

The distribution of phospholipids between outer and inner leaflets of the plasma membrane bilayer is asymmetrical [1–4]. The physiological role of this asymmetry and the mechanism whereby it arises have remained to be determined. Acquisition of an appropriate probe to measure phospholipid translocation with high sensitivity and high time-resolution would facilitate related experiments. Radioactive phospholipid probes with normal chain-length acyl chains were used [5], but these are not suitable for translocation kinetic measurements as about 1 h is needed to incorporate the probe into the membrane. Radioactive dilauroylphosphatidylserine and dilauroylphosphatidylcholine needed shorter time to be incorporated into and extracted out of the outer leaflet of the lipid bilayers [6], but required more than 5 min, hence, are not satisfactory for the measurement with high time-resolution. Fluorescent phospholipid probes with a label on the β-acyl chain of phospholipids were also used, and the asymmetrical distribution of PS in erythrocyte membranes was confirmed [7,8]. However, the relatively longer time (more than 30 min) for incorporation and extraction of the probes into and out of biomembranes [8] is not appropriate for kinetic measurement of transbilayer phospholipid translocation. On the other hand, spin probes with a short acyl chain at the 2-position of the glycerol skeleton are useful for such measurements, with a high time-resolution [9–12]. In erythrocyte membranes, the existence of aminophospholipid translocase has been demonstrated using such spin-labeled phospholipids [9–11,13]. Although the electron paramagnetic resonance (EPR) method has the advantage of providing additional information, based on EPR line shape, to the intensity, the sensitivity of the EPR method is not high enough and the chemical instability of the spin labels often hampers quantitative measurements. In our attempt to measure transbilayer phospholipid translocation in some organella membranes, we have indeed learned that, because of its limited sensitivity, the EPR method is very

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Abbreviations: BSA, bovine serum albumin; CDI, *N,N*-carbonyldiimidazole; C5-PC, 2-pentanoyl-phosphatidylcholine; C5-PE, 2-pentanoyl-phosphatidylethanolamine; C5-PS, 2-pentanoyl-phosphatidylserine; DFP, diisopropyl fluorophosphate; lysoPC, lysophosphatidylcholine; NBD-PC and -PS, 7-nitrobenz-2-oxa-1,3-diazole-phosphatidylcholine and phosphatidylserine; RI-PL, radioactive phospholipid probe; TLC, thin-layer chromatography.

difficult to apply for biomembrane samples which are not available in large amount.

Therefore, we planned to develop novel probes that enable the measurement with a higher sensitivity and accordingly synthesized three radioactive phospholipid probes (*C5-PC, *C5-PE, and *C5-PS) with a short acyl chain at the 2-position of the glycerol skeleton. To confirm the applicability of these probes, phospholipid translocation in erythrocyte membranes was measured with the probes, and the results were compared with findings obtained with spin probes. These novel radioactive probes provide a convenient and highly-sensitive approach for measuring translocation kinetics and equilibrium distribution of phospholipids between the outer and inner leaflets of biomembranes. A preliminary acount of this work has appeared in an abstract form [14].

Materials and Methods

Synthesis of phospholipid probes

The scheme used for the synthesis is given in Fig. 1. All the commercially available reagents were used as received unless otherwise stated and the solvents used for the reactions were distilled prior to use. Four types of phospholipase D (type I from cabbage, IV from peanut, VI from *Streptomyces chromofuscus*, and VII from *Streptomyces* species) were obtained from Sigma (St. Louis, MO, USA), and egg lysophosphatidylcholine (lysoPC) was obtained from Avanti Polar Lipids (Pelham, AL, USA).

Analytical and preparative thin-layer chromatography (TLC) was done on precoated silica gel 60 F_{254} and silica gel 60 plates (Merck), respectively, which were viewed by staining with iodine vapor, ninhydrin reagent, or ammonium molybdate reagent. The ninhydrin reagent was made by dissolving 180 mg of ninhydrin in 100 ml ethanol. The ammonium molybdate reagent was prepared as follows: 160 mg copper powder and 500 mg ammonium molybdate were mixed well with 2 ml concentrated H_2SO_4 and 80 ml water and the preparation was left to stand at room temperature for 2 h, after which the undissolved copper powder was filtered off and 6.4 ml concentrated H_2SO_4 was added to the filtrate. ^1H and ^{13}C-NMR spectra were measured on a JEOL JNM GX-270 spectrometer operating at 270 and 68 MHz, respectively. Tetramethylsilane was used as an internal reference.

*Synthesis of *C5-PC.* Sodium salt of [^{14}C]pentanoic acid (41.0 μmol, spec. act. 6.1 mCi/mmol from Sigma) and unlabeled pentanoic acid (82.0 μmol) were mixed and dissolved in 1 ml water and the pH was adjusted to 1.0 with concentrated HCl. The free pentanoic acid was extracted 5-times with 2 ml $CHCl_3$, the chloroform solution was dehydrated with anhydrous sodium sulfate and evaporated, thereby producing a residue of 90 μmol [^{14}C]pentanoic acid (yield 73%) with a specific activity of 2 mCi/mmol.

N,N-carbonyldiimidazole (CDI) 21.4 mg, 132 μmol, was added to the 90 μmol [^{14}C]pentanoic acid and the preparation stirred for 10 min at room temperature under a N_2 atmosphere, 28.3 mg (54 μmol) lysoPC and 200 ml dry $CHCl_3$ were added and the mixture was maintained at 52°C for 20 h with stirring. The reaction was stopped by adding distilled water and the product was purified by column chromatography with silica gel 60 (Merck). The yield of *C5-PC was 20 μmol (37%).

*Synthesis of *C5-PE.* 2 μmol *C5-PC was mixed with 0.1 ml 1 M $CaCl_2$, 0.1 ml 1 M CH_3COONa (pH

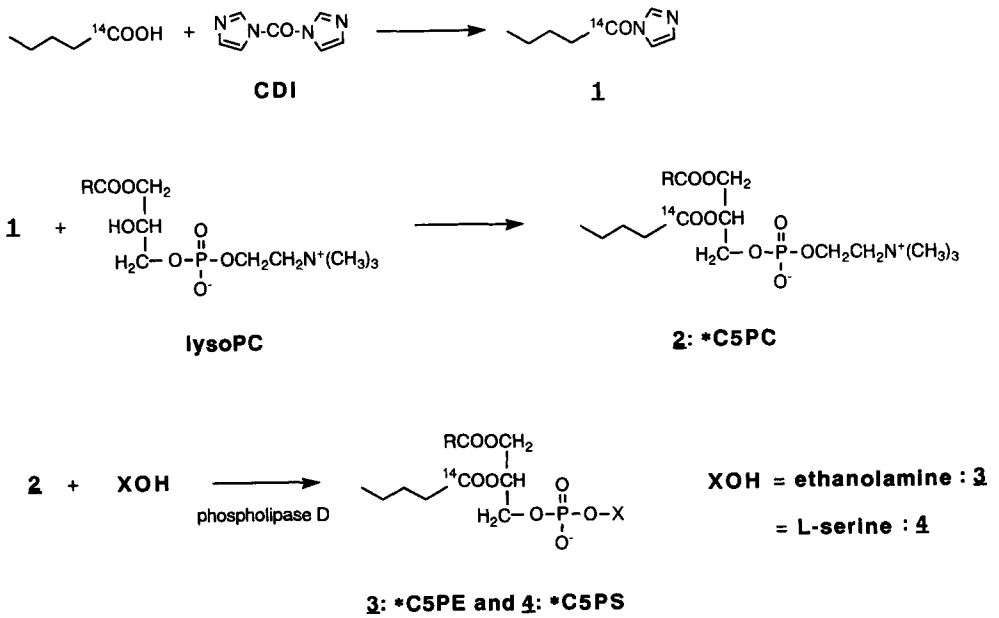

Fig. 1. A scheme for the synthesis of the radioactive phospholipid probes (*C5-PC, *C5-PE and *C5-PS).

5.6), 0.5 ml 50% (w/w) ethanolamine, 0.1 ml phospholipase D (Type VII from *Streptomyces* species, Sigma) solution (50 μg protein/ml) and 0.2 ml H_2O. The mixture was maintained at 45°C for 2 h with vigorous stirring, after which the reaction was stopped by adding 1 ml 100 mM EDTA (pH 7.4). Methanol and $CHCl_3$ (10 ml each) were added to the solution, which was stirred for 10 min, centrifuged at $750 \times g$ for 15 min, the upper (aqueous) layer was removed and the lower layer was evaporated to dryness. The resulting residue was dehydrated by repeated evaporation with dry ethanol and $CHCl_3$. As there was only one spot after TLC of the product, no further purification was done (yield 1.8 μmol, 90%).

*Synthesis of *C5-PS.* 10 μmol *C5-PC was mixed with 0.1 ml 1 M $CaCl_2$, 0.1 ml 1 M CH_3COONa (pH 5.6), 0.5 ml 50% (w/w) L-serine solution, 0.1 ml phospholipase D (Type VII from *Streptomyces* species, Sigma) solution (50 μg protein/ml) and 0.2 ml H_2O. The mixture was maintained at 45°C for 2 h with vigorous stirring and then the reaction was stopped by adding 1 ml 100 mM EDTA (pH 7.4). Methanol and $CHCl_3$ (10 ml each) were added to the solution, which was stirred for 10 min, centrifuged at $750 \times g$ for 15 min, after which the upper (aqueous) layer and unreacted L-serine (precipitate) were removed and the lower layer was evaporated to dryness. The resulting residue was dehydrated by repeated evaporation with dry ethanol and $CHCl_3$, and the crude *C5-PS was purified by preparative TLC using $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (25:15:4:2) as the developing solvent, which produced 2 μmol purified *C5-PS (yield 20%).

Synthesis of spin-labeled PC and PE. Spin-labeled PC and PE with a short spin-labeled acyl chain at the 2-position of the glycerol skeleton were synthesized using a procedure similar to that used for the synthesis of the RI-PLs. 4-Doxyl-levulinic acid was used instead of [^{14}C]pentanoic acid.

Preparation of erythrocytes

Fresh human whole blood was centrifuged 4-times ($1000 \times g$, 5 min) with buffer A containing 145 mM NaCl, 5 mM KCl, 10 mM glucose, and 20 mM Hepes-Na (pH 7.4). The final pellet was suspended in 5 vols. of buffer A, which resulted in a 17% hematocrit and a lipid concentration 0.5 mg/ml.

To prepare ATP-depleted erythrocytes, whole blood was washed as described above, with a modified buffer A containing no glucose or 10 mM deoxyglucose. The suspension of erythrocytes was further incubated for up to 24 h at 37°C in the modified buffer (also supplemented with 0.2 mg/ml streptomycin and 200 IU/ml penicillin). The ATP content was measured by chemiluminescence using an ATP bioluminescent assay kit (Sigma). The luminescence was monitored with a Chem-Glow photometer (Aminco).

Measurement of incorporation efficiency of RI-PLs into erythrocyte membranes

Ethanol solution of the radioactive phospholipid probe (RI-PL) (20 μl, $2 \cdot 10^3$ dpm/μl) was mixed with 5 ml of the erythrocyte suspension (0.5 mg lipid/ml) in buffer B containing 5 mM diisopropyl fluorophosphate (DFP) in addition to the solution of buffer A and the mixture was incubated at 37°C. From time to time a 500 μl sample was taken and centrifuged at $7800 \times g$ for 1 min. The radioactivity of the supernatant (250 μl) was counted in a liquid scintillation counter (LSC 1000, Aloka).

Measurement of phospholipid translocation with RI-PLs

To the erythrocyte suspension (0.5 mg lipid/ml) in buffer B (1.5 ml), 15 μl ethanol solution of the RI-PL was added and the mixture was incubated for 2 min at 37°C, centrifuged at $7800 \times g$ for 1 min and the resultant pellet was resuspended in 1.5 ml of the buffer B and incubated at 37°C. From time to time, a 100-μl aliquot of the suspension was taken, mixed with 200 μl of 10% bovine serum albumin (BSA, essentially fatty acid free, Sigma) and incubated for 1 min on ice. The RI-PL molecules present only in the outer leaflet of the membranes were extracted, using this procedure. The mixture was centrifuged at $7800 \times g$ for 1 min and the radioactivity of the supernatant (200 μl) was counted. Total radioactivity in the 100-μl aliquot was also counted. For the control experiments, the same procedure (except for use of a solution containing no BSA) was also used and the radioactivities thus obtained were subtracted from those obtained by the BSA-extraction procedure.

Measurement of phospholipid translocation with EPR spin probes

To 4 ml of the suspension of erythrocytes (2.5 mg lipid/ml) in buffer B, 500 μl of the suspension of spin probe (2% of total phospholipid) in buffer A was added, and the mixture was incubated at 37°C. From time to time, a 300 μl aliquot of the suspension was taken, centrifuged at $7800 \times g$ for 5 min, and the resultant pellet was resuspended in 180 μl of 10% BSA. After incubating for 1 min on ice, the suspension was centrifuged at $7800 \times g$ for 1 min. To 90 μl of the supernatant, 10 μl of 100 mM potassium ferricyanide was added and EPR spectrum was measured.

Results and Discussion

Synthesis of radioactive phospholipid probes

We examined many reaction conditions using unlabeled compounds before synthesizing the radioactive phospholipid probes. Unlabeled C5-PC was synthesized on a relatively large scale using 800 μmol lysoPC and 340 μmol pentanoic acid. The yield was 65%,

which exceeds the spin-labeled PC (14%) obtained by Boss et al. [15], who used a similar, but not identical, CDI method. The two major modifications and improvements we developed were as follows: (1) we mixed CDI directly with pentanoic acid, whereas Boss et al. dissolved it in 0.4 ml $CHCl_3$; (2) we allowed CDI and pentanoic acid to react for 10 min, which was 20 min shorter than that used by Boss et al. These modifications increased the amount of the active intermediate produced, which in turn provided an increased yield. The synthesized C5-PC was analyzed by ^1H and ^{13}C-NMR and the spectra were typical of phosphatidylcholine [16,17]: ^1H-NMR ($CDCl_3$) δ (ppm) 0.89 (6H, q, $J = 7.4$ Hz, terminal CH_3), 1.25 (26–27H, m, $-CH_2-$), 1.58 (4H, m, CH_2CCO), 2.30 (4H, q, $J = 7.9$ Hz, CH_2CO), 3.35 (9H, s, $N(CH_3)_3$), 3.78 (2H, br s, CH_2N), 3.93 (2H, br t, CH_2OP glycerol), 4.13 (1H, dd, $J = 7.3$, 11.9 Hz, CH_2OCO glycerol), 4.28 (2H, br s, $POCH_2$ choline), 4.38 (1H, dd, $J = 2.5$, 12.5 Hz, CH_2OCO glycerol), 5.19 (1H, m, CHOCO glycerol); ^{13}C-NMR ($CDCl_3$) δ (ppm) 13.7 and 14.1 (terminal CH_3), 22.2, 22.7, 25.0, 27.0, 29.7, 31.9, and 34.1 ($-CH_2-$), 54.3 ($N(CH_3)_3$), 59.4 ($POCH_2$ choline), 63.0 ($\underline{C}H_2OCO$ glycerol), 63.5 (CH_2OP glycerol), 66.3 (CH_2N), 70.6 ($\underline{C}HOCO$ glycerol), 173.2 and 173.6 (C=O).

The efficiencies of four different phospholipases D to catalyze the conversion of C5-PC to C5-PE or C5-PS were compared by TLC analyses of the reaction products. Fig. 2A shows a chromatogram stained with iodine vapor after development with $CHCl_3/CH_3OH/H_2O$ (65:25:4). Fig. 2B shows the findings in the case of staining with the ninhydrin reagent with which phospholipids containing amino groups can be detected. Fig. 2C shows bands stained with the ammonium molybdate reagent with which all phospholipids can be detected. Fig. 2A–C shows clearly that the phospholipase D from *Streptomyces* species is the best for the exchange reaction used to synthesize C5-PE and C5-PS. The high conversion of PC to PS with normal acyl-chain length catalyzed by phospholipase D from *Streptomyces* species has been reported [18–20]. In view of this result we used phospholipase D from *Streptomyces* species for the synthesis of radioactive *C5-PE and *C5-PS.

Comfurius et al. improved the exchange reaction by using a one-phase system in the presence of a detergent [20]. We examined the effect of the detergent octylthioglucoside on the synthesis of NBD-PS by the exchange reaction from NBD-PC (a more hydrophobic lipid than C5-PC). The yield in the presence of octylthioglucoside was 61%, whereas that in its absence was 55%. As the difference between the yields was small, removal of the detergent from the product was difficult, and because C5-PC is more water-soluble than NBD-PC, we used the procedure without detergent to synthesize radioactive *C5-PE and *C5-PS.

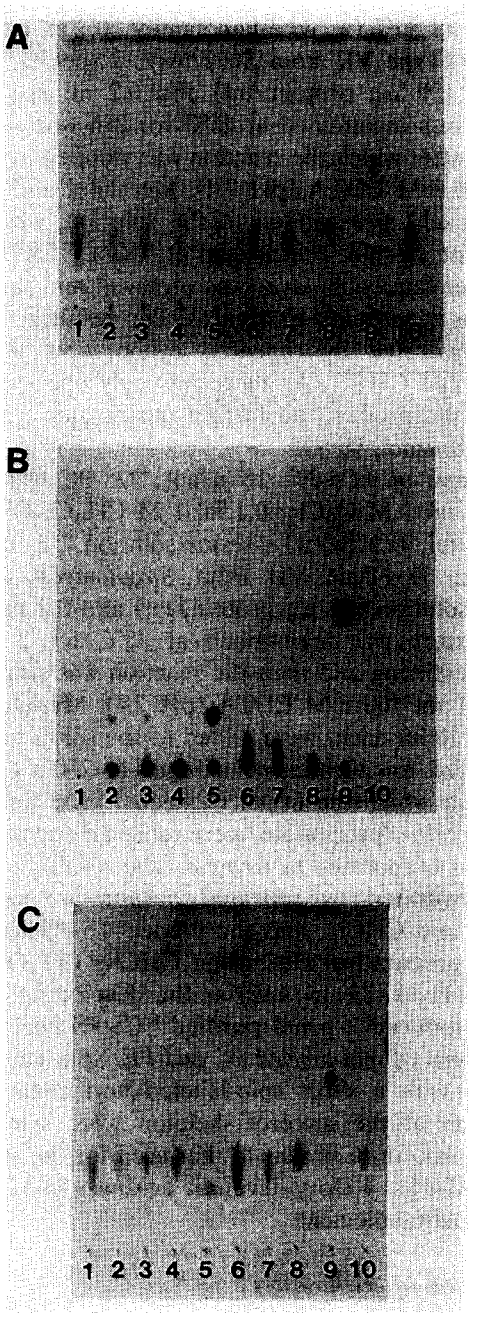

Fig. 2. Conversion of C5-PC to C5-PE and to C5-PS by four kinds of phospholipases D as revealed on TLC. (A) Stained with iodine vapor. (B) Stained with ninhydrin reagent. (C) Stained with ammonium molybdate reagent. Lanes 1 and 10, C5-PC (starting material); lanes 2–5, reaction products for the synthesis of C5-PS; lanes 6–9, reaction products for the synthesis of C5-PE. The phospholipases D are from cabbage (lanes 2 and 6), peanut (lanes 3 and 7), *Streptomyces chromofuscus* (lanes 4 and 8) and *Streptomyces* species (lanes 5 and 9).

The synthesized RI-PLs were analyzed by autoradiography with a Bioimage Analyzer (BAS2000, Fuji Photo Film, Tokyo, Japan) after separation by TLC (silica gel 60) with a developing solvent, $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (25:15:4:2). All the RI-PLs

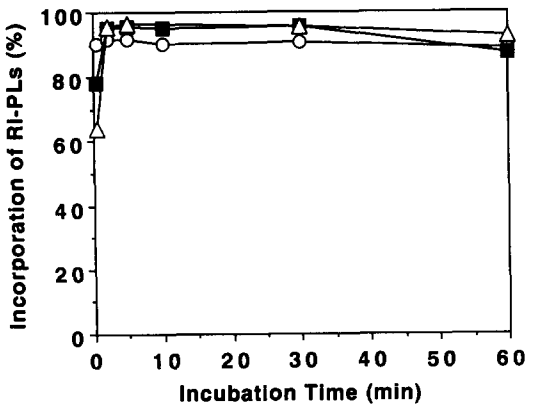

Fig. 3. Time-course of incorporation of RI-PLs into the erythrocyte membranes. Ethanol solution of *C5-PS (○), *C5-PE (■), or *C5-PC (△) was mixed with the erythrocyte suspension in 145 mM NaCl, 5 mM KCl, 10 mM glucose, 20 mM Hepes-Na (pH 7.4) and 5 mM diisopropyl fluorophosphate (DFP) and the mixture was incubated at 37°C. From time to time an aliquot was taken and centrifuged at 7800× *g* for 1 min. The fraction of the radioactivity of total minus supernatant to the total radioactivity is shown.

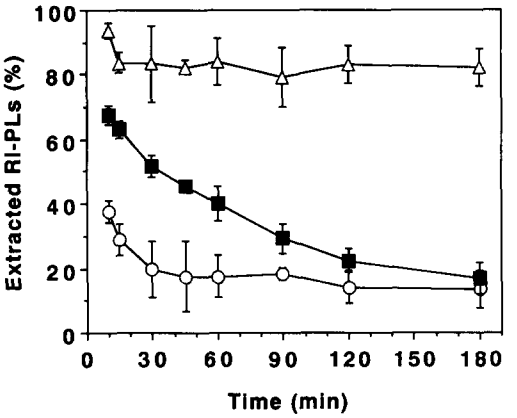

Fig. 4. Change in time of the fraction of BSA-extracted RI-PLs in the total RI-PLs incorporated into the erythrocyte membranes. See the text for detailed experimental procedures. (○), *C5-PS; (■), *C5-PE; (△), *C5-PC.

were pure with one radioactive spot. The acylation reaction for the synthesis of *C5-PC proceeded to about 80%, which was monitored by TLC separation of the reaction mixture, whereas the yield after purification was 37%. The conversion from *C5-PC to *C5-PE and *C5-PS proceeded to 97% and 46%, respectively, whereas the yields of the purified materials were 90% and 20%, respectively. These results suggest that separation is critical to improve the efficiency of the small-scale synthesis of these radioactive phospholipid probes.

Measurement of phospholipid translocation in erythrocyte membranes

Fig. 3 shows the incorporation of the RI-PLs in the erythrocyte membranes. In all the RI-PLs examined, more than 90% of the probe molecules added were incorporated in the erythrocyte membranes within 2 min and the incorporation was stable for more than 30 min. Based on this result we set the incubation time as 2 min in the following translocation measurements.

Fig. 4 shows the time-course of the fraction of BSA-extracted RI-PLs in the total RI-PLs. The value essentially represents the amount of RI-PLs in the outer leaflet of the erythrocyte membranes at the time of BSA extraction. The amount of extracted *C5-PS rapidly decreased and reached a steady state of about 15%. *C5-PE had a smaller decreasing rate than *C5-PS but a similar level was finally reached. On the other hand, *C5-PC took a different time-course from the aminophospholipid probes (*C5-PS and *C5-PE). *C5-PC could be extracted more than 80%, even after 3 h incubation. These results are comparable to the results obtained using spin-labeled phospholipid probes [9,13].

The aminophospholipid translocation is proposed to be mediated by a translocase that requires ATP for function [5,9,21]. Therefore, we measured the effect of ATP depletion on phospholipid translocation in erythrocytes. Fig. 5 shows the time-course of ATP depletion in different incubation media. With incubation for 24 h in either of the buffers (containing no glucose or containing deoxyglucose instead of glucose in buffer A) intracellular ATP was depleted and little hemolysis was evident after 24 h of incubation.

Change in time of the fraction of BSA-extractable RI-PLs in the total RI-PLs incorporated in the ATP-depleted erythrocyte membranes is shown in Fig. 6. Although the extractable amount was not the same among the RI-PLs, the amount was fairly constant throughout the incubation period, for all the probes examined. This means that no RI-PL was translocated quickly from outer to inner leaflet of the ATP-depleted

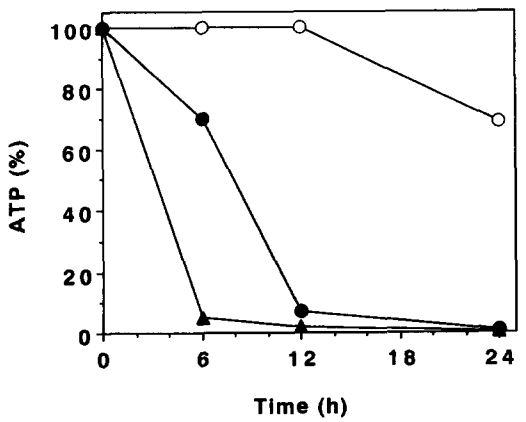

Fig. 5. Time-course of decrease in intracellular ATP in different incubation media. The erythrocytes were incubated at 37°C in control buffer (○) containing 145 mM NaCl, 5 mM KCl, 10 mM glucose, 20 mM Hepes-Na (pH 7.4), 0.2 mg/ml streptomycin and 200 IU/ml penicillin. (●), Glucose was omitted from the control buffer; (▲), deoxyglucose was used instead of glucose.

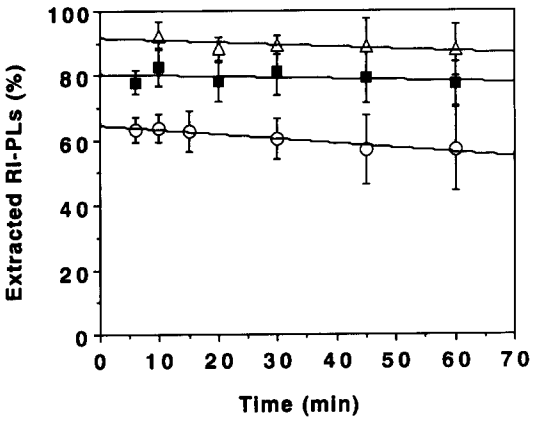

Fig. 6. The proportion of BSA-extracted RI-PLs to the total RI-PLs in the erythrocytes where intracellular ATP was depleted by the preincubation in glucose free buffer for 24 h as shown in Fig. 5. (○), *C5-PS; (■), *C5-PE, (△), *C5-PC.

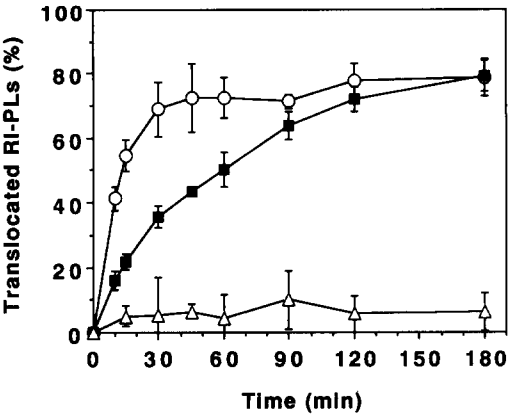

Fig. 7. Phospholipid translocation from the outer to the inner leaflet of plasma membranes of erythrocyte as probed with RI-PLs. The fraction of the probes translocated from outer to inner leaflet was estimated using the equation presented in the text. (○), *C5-PS; (■), *C5-PE; (△), *C5-PC.

erythrocytes membranes and confirms that the translocation of aminophospholipids in erythrocyte membranes is mediated by an ATP-dependent process. Therefore, the proposal that aminophospholipid translocase is present in erythrocytes is given support [9].

Extrapolating the time-course of Fig. 6 to time zero, the amount of maximum extractable probe can be estimated; that is 64%, 80% and 92% for *C5-PS, *C5-PE and *C5-PC, respectively. These values are not likely to be 100% because equilibrium of the probes between the erythrocyte membranes and BSA does not lie completely to BSA. The difference among the three probes indicates a possible interaction of the probe with some membrane components, for example membrane proteins [22]. It is important to estimate the contribution of these effects in using this type of measurement (see Table II).

Taking into account these amounts of BSA-unextractable probes, the translocation of each RI-PL from outer to inner leaflet was calculated by the following equation,

$$I(t)(\%) = (1 - E(t)/E_{max}) \times 100 \quad (1)$$

where $I(t)$ is the fraction of the translocated probe in the translocatable probe at time t, $E(t)$ is the fraction of the probe extracted by BSA in the total amount of the probe at time t, and E_{max} is the fraction of the extractable probe in the total amount of the probe at time zero (64%, 80% and 92% for *C5-PS, *C5-PE and *C5-PC, respectively). The result is shown in Fig. 7. Again a fast translocation rate for *C5-PS, moderate translocation rate for *C5-PE, and slow translocation rate for *C5-PC are evident. The plateau values at 3 h for the fraction of the translocated probes are 78%, 79% and 7% for PS, PE and PC, respectively.

Effects of sulfhydryl reagents on the translocation reaction were examined by preincubating the erythrocytes with 5 mM *N*-ethylmaleimide or 20 mM iodoacetamide for 30 min. Both PS and PE translocation were inhibited by these treatments, whereas PC translocation was accelerated (Table I), the acceleration of PC translocation by inhibition of aminophospholipid translocase has also been noted in synaptosomes from electric organ of *Torpedo marmorata* [23]. With treatment with SH-reagents the difference in the translocation among the three phospholipids practically disappeared. Thus, these reagents seem to inhibit translocase specific for aminophospholipids but do not inhibit non-specific translocation in erythrocytes.

Comparison of RI probes with spin probes

The kinetic parameters for the translocation reaction obtained with the RI-PLs were compared with values obtained with spin-labeled phospholipid probes (Table II). Similar equilibrium distribution (inner leaflet (%)) and half-time ($t_{1/2}$) between the RI-label and spin-label methods were obtained. This result indicates that the RI probes well represent the translocation

TABLE I

Inhibition of translocation by N-ethylmaleimide and iodoacetoamide

Translocation of the probes from outer to inner leaflet of the erythrocyte membranes was measured after incubation with 5 mM *N*-ethylmaleimide or 20 mM iodoacetoamide. The proportion of translocated probes to the total probes at 15 min is shown.

Treatment	Translocation (%)		
	PS	PE	PC
None	69	36	4
5 mM *N*-ethylmaleimide	35	29	34
20 mM Iodoacetoamide	30	13	32

TABLE II

Comparison of RI label and spin-label methods

Method	Inner leaflet [a] (%)			$t_{1/2}$ [b] (min)			Minimum amount of sample required (mg lipid)
	PS	PE	PC	PS	PE	PC	
RI label [c]	78±6	79±5	7±8	8	42	–	0.05
	[86±6	81±5	20±8	10	70	–] [d]	
Spin label [e]	–	82	35	–	60	–	0.7
Spin label [f]	90±4	80±5	30±7	5	60	480	–

[a] Fraction of phospholipid probes translocated into inner leaflet in 3 h.

[b] Time taken for translocation of a half amount of phospholipid probes from outer to inner leaflet.

[c] This work. The values were obtained taking into account the amount of unextractable RI-PLs. See text.

[d] Based on the same experimental data as above, these were obtained ignoring unextractable RI-PLs to compare the values directly with those obtained by the spin-label method.

[e] This work. A limited number of experiments was done.

[f] Values estimated based on the report by Morrot et al. [11].

kinetics and equilibrium distribution of natural phospholipids in erythrocyte membranes. The amount of samples required to obtain one experimental data point for the RI-label method was 50 μg, while that for the spin-label method was 700 μg, which indicates that the RI-label method is more than 10-times as sensitive as the spin-label method. An even higher sensitivity can probably be achieved in the RI-label method if use is made of probes with a higher specific radioactivity. Such probes can be synthesized by using [^{14}C]pentanoic acid without dilution with unlabeled pentanoic acid (3-fold dilution was done in this work). The RI method may be less disturbing to the lipid bilayer of biomembranes, since there is no bulky group present in the spin-label. The present study shows that the RI-PLs have the advantages of high sensitivity, chemical stability and absence of bulky groups, advantages which facilitate measurements of phospholipid translocation in a wide range of biomembranes. Since the spin-label method, based on EPR line shape, provides information other than the intensity, the high-sensitive RI-label method and the spin-label method will be complementary. Application of these novel radioactive probes to the measurement of transbilayer phospholipid translocation in synaptosomal and synaptic vesicle membranes from electric organ of the electric ray, *Narke japonica*, is currently under way.

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