Nonenzymatic synthesis of glycerolipids catalyzed by imidazole

Eric Testet,[1,*] Malika Akermoun,* Miyuki Shimoji,[†] Claude Cassagne,* and Jean-Jacques Bessoule*

UMR 5544/ESTBB,* CNRS-Université Victor Segalen Bordeaux 2, 146 rue Léo Saignat, 33076 Bordeaux Cedex, France; and Institute of Environmental Medicine,[†] Division of Biochemical Toxicology, Karolinska Institute, Box 210, S-17177, Stockholm, Sweden

Abstract **Imidazole catalyzed acylations of lysolipids by acyl-CoAs in water at room temperature and at a pH close to neutrality. In the presence of oleoyl-CoA and either lysophosphatidylcholine, 1-palmitoyl-*sn*-glycero-3-phosphocholine (LPC); lysophosphatidylglycerol, monoacyl-*sn*-glycero-3-phosphoglycerol; lysophosphatidyl acid, 1-oleoyl-*sn*-glycero-3-phosphate; lysophosphatidylserine, monoacyl-*sn*-glycero-3-phosphoserin; or lysophosphatidylethanolamine, monoacyl-*sn*-glycero-3-phosphoethanolamine, the corresponding phospholipids were synthesized. Similarly, the use of lyso-platelet activating factor, an ether analog of LPC, yielded the formation of 1-*O*-alkyl-2-oleoyl-*sn*-glycero-3-phosphocholine. In the presence of LPC, an imidazole-catalyzed synthesis of phosphatidylcholine (PC) occurred when medium, long, and very long chain acyl-CoAs were added. With hydroxyacyl-CoA, a similar PC synthesis was obtained. The process described in the present paper appears to offer several potential applications of interest for the synthesis of glycerophospholipids and triglycerides with labeled and/or an unusual or fragile fatty acid, or when suitable acyltransferases have not yet been described in the literature and/or are not commercially available. The method described is very safe and simple since lipids can be synthesized in tubes containing 0.7% imidazole in water, and left for a few hours at room temperature on the bench.**—Testet, E., M. Akermoun, M. Shimoji, C. Cassagne, and J-J. Bessoule. **Non-enzymatic synthesis of glycerolipids catalyzed by imidazole. *J. Lipid Res.* 2002.** 43: **1150–1154.**

Supplementary key words acyl-CoA • lysolipid • acylation • chemical method • omega hydroxypalmitoyl-CoA

Lipids are not only efficient reserves for organisms and major components of cell membranes, but are also active compounds involved in a wide range of cellular responses. Studies in this field generally require a wide range of very specific lipid derivatives in small amounts and of high purity, and are ideally available as labeled products (1–2). Unfortunately, such molecules are often not commercially available and must be synthesized chemically or enzymatically (when possible) before the experiments.

An overview of the literature shows that lipids of interest are generally synthesized as follows: the first step is an enzymatic hydrolysis catalyzed by various lipases (3–7). The lysolipids synthesized in this way are then purified and reacylated with the fatty acid of interest. Chemical and enzymatic methods are currently available for the reacylation step. Frequently (8–10), chemical methods involve the use of fatty acyl chloride, anhydride, or imidazolide, which are usually dissolved in a water-insoluble organic phase. However, the experimental conditions used for such chemical biosynthesis (e.g., temperature, pH) may damage some fragile reaction products. Methods based on enzymatic synthesis (7) suffer from other disadvantages: when not commercially available, the purification of an enzyme devoid of contamination is, when possible, tedious and time-consuming, whereas a crude enzyme preparation may contain significant amounts of endogenous fatty acids or their activated form, acyl-CoA, which may lead to the synthesis of undesirable components. In addition, the use of unusual fatty acids, such as branched or substituted ones or those with varying chain length, is often limited by the specificity of available acyltransferases.

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Abbreviations: C16-OH, omega-hydroxypalmitic acid; LPA, lysophosphatidyl acid, 1-oleoyl-*sn*-glycero-3-phosphate; LPAF, lyso-platelet activating factor, 1-*O*-alkyl-*sn*-glycero-3-phosphocholine; LPC, lysophosphatidylcholine, 1-palmitoyl-*sn*-glycero-3-phosphocholine; LPE, lysophosphatidylethanolamine, monoacyl-*sn*-glycero-3-phosphoethanolamine; LPG, lysophosphatidylglycerol, monoacyl-*sn*-glycero-3-phosphoglycerol; LPS, lysophosphatidylserine, monoacyl-*sn*-glycero-3-phosphoserine; NAPE, *N*-acyl phosphatidylethanolamine, diacyl-*sn*-glycero-3-phospho [*N*-octadecenoyl]-ethanolamine; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PS, phosphatidylserine.

[1] To whom correspondence should be addressed.
e-mail: eric.testet@estbb.u-bordeaux2.fr

In the present paper, a non-enzymatic method for the synthesis of glycerolipids is described; acylation of various lysolipids by various acyl-CoAs occurred in the presence of imidazole in water at room temperature and at a pH close to neutrality. This process can be useful, for example, to synthesize glycerolipids with labeled and/or an unusual or sensitive fatty acid, or when suitable enzymes have not yet been described in the literature and/or are not commercially available.

MATERIALS AND METHODS

Materials

TLC plates were HPLC silica gel 60 plates (Merck 60 F 254). [1-^{14}C]oleyl-CoA (specific radioactivity, 55 mCi/mmol), [1-^{14}C]-acetyl-CoA (specific radioactivity, 52 mCi/mmol), and [1-^{14}C]1-palmitoyl-*sn*-glycerol-3-phosphocholine (57 mCi/mmol) were purchased from New England Nuclear, Boston, MA. All other reagents were from Sigma (St. Louis, MO). Labeled glycerolipids were identified and quantified using a PhosphorImager (Molecular Dynamics, Amersham Pharmacia Biotech, Orsay, France).

Methods

Omega-hydroxy fatty acyl-CoA was obtained as follows: 10 mg of C16-OH (omega-hydroxypalmitic acid) were diluted in 1 ml of chloroform-methanol (9:1, v/v) and dried under nitrogen. A quantity of 0.2 ml of NH_4OH (2 M) was then added, and after incubation at 60°C for 30 min NH_3 was blown down under nitrogen before 2.5 ml of water and 1 ml of 10% Triton X-100 were added (final volume 5 ml). The incubation was then carried out in the presence of C16-OH (1 mM final), 0.12M Tris-HCl (pH 7.5), 8 mM ATP, 16 mM phosphoenolpyruvate, 20 mM $MgCl_2$, 8 mM KCl, pyruvate kinase, myokinase, and acyl-CoA synthetase from Pseudomonas (0.01 unit, Sigma A-2777). After 10 min at 30°C, CoASH (0.5 mM) was added to start the reaction. After 1 h of incubation, unreacted free fatty acids were extracted by HCl/isopropanol and acyl-CoAs by water-saturated butanol. The amount and the purity of the product (acyl-CoA) were determined after chromatography on TLC plates using butanol-acetic acid-water (5:2:3, v/v/v) as solvent. This acyl-CoA as well as other unlabeled acyl-CoA (from Sigma) were incubated with labeled lyso-PC in the presence of imidazole as indicated in the legend of the Fig. 7.

Experiments carried out in the presence of unlabeled lysolipids and labeled oleoyl-coenzyme A were carried out as follows: lysolipid samples (5 nmol) dissolved in chloroform-methanol (2:1, v/v) were evaporated to dryness and then dispersed by sonication (10 min) in 70 μl water. Routinely, 20 μl of 0.5 M imidazole and 10 μl [^{14}C]oleoyl-Coenzyme A (0.5 nmol) were added and incubated for 3 h at 30°C (final volume 100 μl).

After incubation, 2 ml of chloroform-methanol (2:1, v/v) and 500 μl of water were added to the mixture in order to stop the reaction and to extract lipids. After phase separation, the organic phase was isolated and 2 ml of chloroform were added to the remaining aqueous phase. After a new phase separation, the organic phases were pooled and evaporated to dryness. Lipids were then suspended in 50 μl chloroform-methanol (2:1, v/v). Polar lipids were resolved by one-dimensional TLC using the solvent system described by Vitiello and Zanetta (11). Identification of radioactive spots was performed by co-migration with unlabeled lipid standards visualized under iodine vapors. The lipid labels were quantified as described above.

Phospholipase A_2 assays were carried out as described previously (12). Briefly, after chromatography the silica gel zones corresponding to PC (neosynthesized PC and commercial 1-palmitoyl, 2-[^{14}C]linoleoyl-PC) were scraped off the plates and sonicated for 15 min in 0.2 ml of 50 mM Tris-HCl, pH 8.9, and 5 mM $CaCl_2$. Reactions were started by the addition of 0.2 units of phospholipase A_2 (Sigma P-8913). Incubations were performed for 15 min at 37°C. After incubation, 2 ml chloroform-methanol (2:1, v/v) were added in order to stop reactions and to start lipid extraction. The organic phase was washed with 1 ml of 0.2 M H_3PO_4, 1 M KCl. The aqueous phase was re-extracted by 2 ml of chloroform. Both of the organic phases were combined, evaporated, and lipids were redissolved in a minimal volume of chloroform-methanol (2:1, v/v). Lipids were resolved by HPLC as described above. After chromatography, the label associated with fatty acids and with lyso-PC was determined as described above.

Alternatively, phospholipase A2 assays were carried out for 15 min at 37°C using 0.25 mg of NAPE as substrate. After incubation, lipids were extracted (as described above) and resolved by HPTLC using two different systems successively: chloroform-methanol-NH4OH-water (65:25:0.9:3; v/v/v/v) and chloroform-methanol-acetic acid-water (40:20:5:0.5; v/v/v/v). The mobility (Rf, Rf) of NAPE (0.73, 0.96), lyso-NAPE (0.62, 0.93), and PE (0.55, 0.81) were then compared with the mobility of lipids extracted from assays carried out with labeled oleoyl-CoA, LPE, and imidazole.

RESULTS AND DISCUSSION

The ability of imidazole to catalyze the non-enzymatic synthesis of glycerolipids was studied by using labeled LPC (0.3 nmol) and oleoyl-CoA (5 nmol) as substrates. The results are shown in **Fig. 1**. In the absence of imidazole, a single major radioactive spot co-migrating with [^{14}C]LPC was present. In contrast, in the presence of various amounts of imidazole, a spot co-migrating with PC was de-

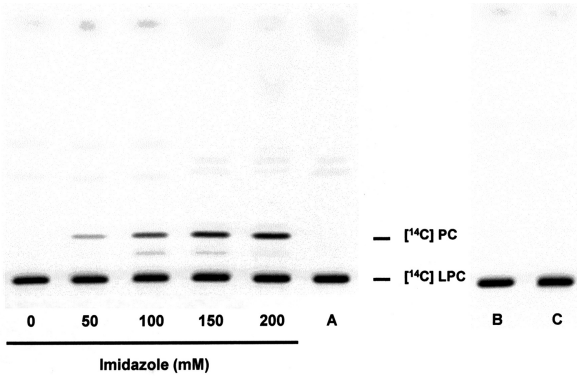

Fig. 1. Non-enzymatic synthesis of phosphatidylcholine in water as a function of imidazole concentration. Labeled lysophosphatidylcholine, 1-palmitoyl-*sn*-glycero-3-phosphocholine (LPC) (0.3 nmol) was incubated for 120 min at 30°C with 5 nmol oleoyl-CoA with imidazole concentrations varying from 0 to 200 mM. The final volume was 100 μl. In control experiments, oleoic acid (5 nmol) was used instead of oleoyl-CoA (line A), and 100 mM glycine-NaOH buffer pH 10 (line B) or 100 mM Tris-HCl buffer pH 10 (line C) instead of imidazole. After incubation, lipids were extracted and purified by TLC, as described in Materials and Methods.

tected. Its label increased as a function of the imidazole concentration. A minor upper spot identified as ^{14}C free fatty acid was detected both in the absence and in the presence of imidazole. Its formation resulted from the hydrolysis of the [^{14}C]LPC during the incubation process. Minor and unidentified spots were also observed, but since they represented less than 1% of [^{14}C]PC, they were not further characterized. When free oleic acid (control A) (even in the presence of various amounts of CoA) or oleic acid methyl ester (not shown) was used as a substrate instead of oleoyl-CoA, no PC synthesis occurred.

The synthesis of PC occurred in water in the presence of 100 mM imidazole. Under these conditions, the pH of the incubation medium was 10. In the absence of imidazole but in the presence of 100 mM glycine-NaOH pH 10 buffer (Fig. 1, control B) or 100 mM Tris-HCL pH 10 buffer (Fig. 1, control C), no PC synthesis occurred. This result clearly shows that the acylation of the LPC molecules evidenced in Fig. 1 was catalyzed by imidazole itself, and not because of the basic pH of the incubation medium. Our finding is close to that made by Burt and Silver (13), who described an imidazole-catalyzed transfer of the acetyl group from acetyl-CoA to choline, and the subsequent formation of acetylcholine. In addition, under the experimental conditions described in the legend of Fig. 1, the formation of PC from 0.5 nmol of LPC and 5 nmol of oleoyl-CoA reached 30 pmol/h (i.e., 20% of the LPC molecules were acylated after 3 h incubation). By taking into account the dilution factors of the various reagents, this rate of PC synthesis is of the same order as that obtained by Burt and Silver (13). The yield of the reaction may appear quite low by comparison with some chemical processes, but several points should be noted: *i*) the present method is very safe and simple since lipids can be synthesized in tubes containing 0.7% imidazole in water, and left for a few hours at room temperature on the bench; *ii*) higher rates were observed when lysolipids other than lyso-PC were used (see below, Figs. 5 and 6); *iii*) the goal of the present paper was not to optimize the yields of the various reactions, but it is possible according to the nature of the lipids that a team might set out to synthesize. For example, the yield of PC synthesis can be increased by increasing not only the imidazole concentration (Fig. 1) but also the incubation time and/or the substrate concentration, as shown in **Fig. 2A, B**. For example, in the presence of 15 nmoles of oleoyl-CoA, 40% of lyso-PC (10 nmoles) was acylated after a 5 h incubation. Nevertheless, when the incubation time was increased, some transacylation occurred. Indeed, after 5 h and 24 h incubation of labeled oleoyl-CoA with unlabeled lyso-PC and 100 mM imidazole, followed by the purification of the reaction product (PC) and its further hydrolysis by phospholipase A_2, we found that 8% and 20–25%, respectively, of the label was associated with lyso-PC and 92% and 75–80% with free fatty acids (100% of the label was associated with free fatty acids in control experiments carried out by hydrolyzing 1-palmitoyl, 2-[^{14}C]linoleoyl-PC under the same experimental conditions). In other words, after 5 h and 24 h incubation of labeled oleoyl-CoA and unlabeled lyso-PC with 100 mM imidazole, 8% and 20–25% of the label respectively was associated with the *sn*-1 position of the PC synthesized.

We further determined whether the acylation of lyso-PC molecules could occur at a pH close to the neutrality. To constitute a pH range, the incubation media were buffered with Tris-HCl 100 mM. Whatever the pH used in a range from pH 7.0 to pH 10.0, no formation of PC occurred in the absence of imidazole (**Fig. 3**). As expected, in the presence of 100 mM imidazole, the pH shift was progressively greater as pH values neared neutrality. Moreover, in agreement with Jencks and Carriuolo (14), who suggested that the active form of imidazole in the catalysis of acetyl transfer reactions is the free base, it was observed (Fig. 3) that the acyl transfer efficiency increased with pH values. Nevertheless, it should be noted that the glycerolipid synthesis also occurred at pH values as low as 7.8, so this imidazole-catalyzed lipid synthesis can also be obtained with molecules that would be sensitive at alkaline pH. This finding is of potential interest.

To analyze the effect of the buffer ionic strength on the non-enzymatic reaction, labeled LPC was incubated with

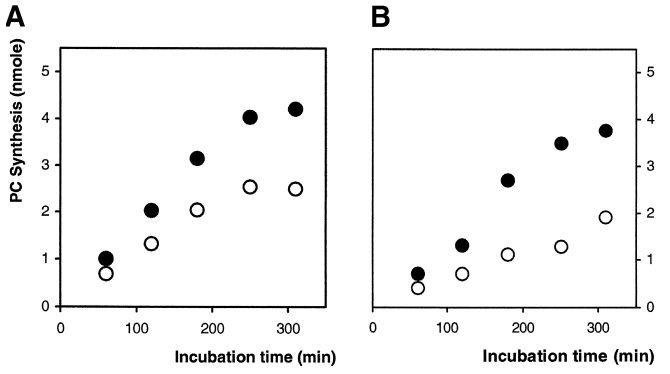

Fig. 2. Non-enzymatic synthesis of phosphatidylcholine as a function of time and substrate concentrations. A: Five nanomoles (open circles) or 15 nmoles (closed circles) of labeled LPC were incubated at 30°C for various times with 30 nmoles of oleoyl-CoA in 100 mM imidazole (final volume 0.1ml). B: Five nanomoles (open circles) or 15 nmoles (closed circles) of oleoyl-CoA were incubated at 30°C for various times with 10 nmoles of labeled LPC in 100 mM imidazole (final volume 0.1ml).

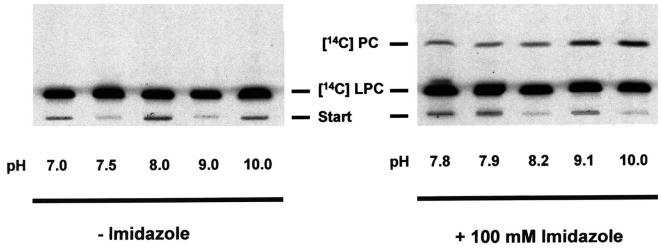

Fig. 3. Non-enzymatic synthesis of phosphatidylcholine as a function of pH. Labeled LPC (0.3 nmol) was incubated with 5 nmoles of oleoyl-CoA in the absence (A) or in the presence (B) of 100 mM imidazole in a 100 mM Tris-HCl buffer, with pH varying from pH 7.0 to pH 10.0. Other conditions as described in Materials and Methods.

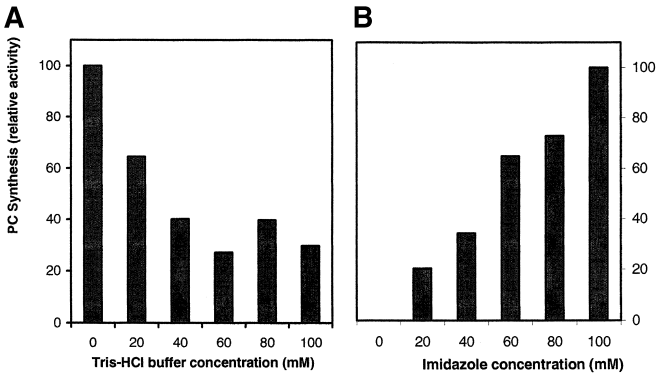

Fig. 4. Non-enzymatic formation of phosphatidylcholine as a function of the buffer and imidazole concentrations. Labeled LPC (0.3 nmol) was incubated for 120 min at 30°C with 5 nmol oleoyl-CoA, and (A) 100 mM imidazole, and various concentrations of Tris-HCl buffer pH 10 or (B) 100 mM Tris-HCl buffer pH 10 and imidazole concentrations varying from 0 to 100 mM (final volume 100 μl). After incubation, lipids were extracted and separated by TLC as described in Materials and Methods.

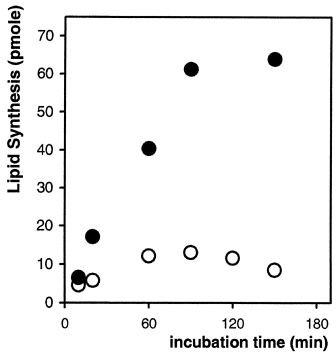

Fig. 6. Label incorporated into the products following the imidazole-catalyzed acylation of lyso-PE as a function of time. Five nanomoles LPE were incubated at 30°C for various times with 0.3 nmole of labeled oleoyl-CoA in 100 mM imidazole (final volume 0.1 ml). After incubations, the radioactivity incorporated into PE (open circles) and NAPE (closed circles) was determined as described in Materials and Methods.

oleoyl-CoA in the presence of 100 mM imidazole and various concentrations of Tris-HCl pH 10. The results (**Fig. 4A**) show that an increase in the buffer concentration induced a decrease in the PC synthesis. Moreover, as in water (Fig. 1), in the presence of 100 mM Tris-HCl pH 10 the rate of PC synthesis increased with the imidazole concentration (Fig. 4B).

The effect of the nature of the acyl acceptor on the imidazole-catalyzed lipid synthesis was further studied by using labeled oleoyl-CoA and various lysolipids. In the absence of lysolipid, no product was detected (not shown), clearly indicating that imidazole is not a substrate for acylation. In the presence of lysophosphatidylglycerol, monoacyl-*sn*-glycero-3-phosphoglycerol (LPG); lysophosphatidyl acid, 1-oleoyl-*sn*-glycero-3-phosphate (LPA); lysophosphatidylserine, monoacyl-*sn*-glycero-3-phosphoserine (LPS); and lysophosphatidylethanolamine, monoacyl-*sn*-glycero-3-phosphoethanolamine (LPE), an incorporation of the acyl moiety of oleoyl-CoA in the corresponding phospholipids [phosphatidylglycerol (PG), phosphatidic acid (PA), phosphatidylserine (PS), and phosphatidylethanolamine (PE), respectively] occurred (**Fig. 5**). Similarly, the use of lyso-platelet activating factor (LPAF) [an ether analog of lysophosphatidylcholine, 1-palmitoyl-*sn*-glycero-3-phosphocholine (LPC)] yielded the formation of 1-*O*-alkyl-2-oleoyl-*sn*-glycero-3-phosphocholine [an analog of platelet activating factor (PAF)]. We also observed an imidazole-catalyzed synthesis of triolein from oleoyl-CoA plus 1,2-dioleoyl-*sn*-glycerol (not shown). Interestingly, in some assays, additional products were detected. In the presence of LPG, an additional upper spot was observed, likely resulting from the synthesis of diacyl-*sn*-glycero-3-phospho-[1,2-dioleoyl]-glycerol synthesized by the acylation of all LPG hydroxyl groups. Similarly, when LPE was present in the incubation mixture, a major product identified (using pure standards) as *N*-acyl-phosphatidylethanolamine (NAPE) was detected. Under the experimental conditions described in the legend of Fig. 5, the relative amount of lipid synthesized (corresponding to 2-fold and 3-fold the amount of labeled acyl-CoA incorporated into NAPE and diacyl-*sn*-glycero-3-phospho-[1,2-dioleoyl]-glycerol respectively) was 1 for PC,

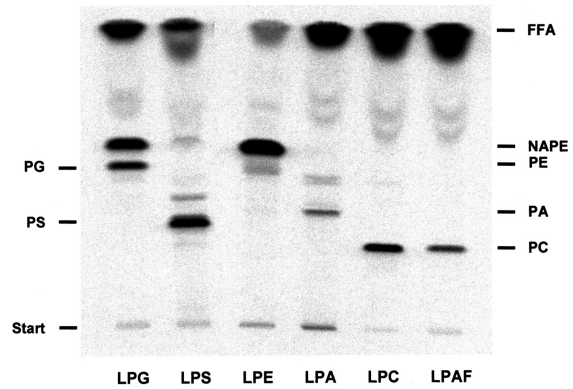

Fig. 5. Non-enzymatic synthesis of various glycerophospholipids. Five nanomoles of unlabeled lysophosphatidylglycerol, monoacyl-*sn*-glycero-3-phosphoglycerol (LPG); lysophosphatidylserine, monoacyl-*sn*-glycero-3-phosphoserine (LPS); lysophosphatidylethanolamine, monoacyl-*sn*-glycero-3-phosphoethanolamine (LPE); lysophosphatidyl acid, 1-oleoyl-*sn*-glycero-3-phosphate (LPA); lysophosphatidylcholine, 1-palmitoyl-*sn*-glycero-3-phosphocholine (LPC); and lyso-platelet activating factor, 1-*O*-alkyl-*sn*-glycero-3-phosphocholine (LPAF) were incubated at 30°C for 3 h with 0.5 nmole of labeled oleoyl-CoA in 100 mM imidazole (final volume 100 μl); other conditions as in Materials and Methods.

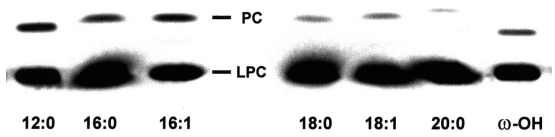

Fig. 7. Non-enzymatic synthesis of phosphatidylcholine with various acyl-CoAs. Labeled LPC (0.5 nmol) was incubated at 30°C for 3 h in 100 mM imidazole with 10 nmol of lauroyl (12:0), palmitoyl (16:0), palmitoleoyl (16:1), stearoyl (18:0), oleoyl (18:1), arachidoyl (20:0), or 16-hydroxy-hexadecanoyl (ω-OH) CoA esters (final volume 0.1 ml).

0.75 and 4.8 for PE and NAPE respectively, 3 for PS, 1.5 and 1 for PG and diacyl-*sn*-glycero-3-phospho-[1,2-dioleoyl]-glycerol respectively, 0.6 for PAF, and 0.25 for PA.

Since oleoyl-CoA reacted with the amino group of ethanolamine, it appears that groups other than hydroxyl can act as acyl acceptors during the imidazole-catalyzed process. To determine whether LPE was acylated to PE before the NAPE synthesis, or whether LPE was acylated to lyso-NAPE and then to NAPE, we carried out experiments with shorter incubation times. As observed in Fig. 5, no spot other than PE and NAPE was detected, and the amount of PE molecules synthesized remained low in comparison with the amount of labeled NAPE (**Fig. 6**). In addition, the shape of the curve expressing the radioactivity incorporated into PE as a function of time is typically of the shape observed for intermediates. Hence, these results strongly suggest that acylation of LPE to PE occurred first, followed by acylation of PE to NAPE, and that the rate of the first reaction was lower than that of the second one (low level of intermediate). The absence of lyso-NAPE suggests that the synthesis of this compound did not occur. Nevertheless, the hypothesis of a lyso-NAPE synthesis occurring at a very lower rate than that of the hypothetical acylation of this compound cannot definitely be ruled out. This absence of labeled lyso-NAPE, as well as the presence of labeled NAPE in the assays after incubations has been checked by HPTLC using two different solvent systems (see the Materials and Methods section).

To analyze the specificity of the imidazole-catalyzed reaction toward acyl-CoAs, experiments were carried out by using labeled LPC and various acyl-CoAs. When [^{14}C]acetyl-CoA was incubated with LPAF or LPC in the presence of 100 mM imidazole, neither 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (PAF) nor phosphatidylcholine were synthesized (data not shown). In contrast, when medium-, long-, and very-long chain acyl-CoAs were used, a PC synthesis occurred (**Fig. 7**). Imidazole also catalyzed the formation of PC containing an omega-hydroxylated fatty acid (Fig. 7). This result underlines the potential applications of the process described in the present paper, since such a synthesis of lipids containing these omega-hydroxylated fatty acids was not possible by using LPC acyltransferase from plastids or LPA acyltransferase from ER membranes (unpublished results). Figure 7 shows some slight differences between the Rf of the various neo-synthesized phosphatidylcholines. As expected, the shorter the added acyl-chain, the lower the Rf. Hence, since the product synthesized by using the omega-hydroxylated fatty acyl-CoA appeared to be one of the most polar PCs, it can be assumed that, for an unknown reason, the omega hydroxy-group was not acylated in the presence of imidazole.

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