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UPTAKE OF THE PHOSPHOLIPASE A_2 INHIBITOR 1-DODECYL 2-[1-^{14}C] OCTANAMIDO-SN-2-DEOXY GLYCERO-3-PHOSPHOCHOLINE BY PERITONEAL MACROPHAGES

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The [^{14}C] phospholipid analogue 1-dodecyl-2-[1-^{14}C] octanamido-sn-2-deoxy glycero-3-phosphocholine was synthetized. With 2 short fatty chains linked by alkyl and amido bonds to positions 1 and 2 of the glycerophosphate backbone, it was an inhibitor of phospholipase A_2 in ionophore A23187-stimulated macrophages. Its uptake by rat peritoneal macrophages and its resistance towards phospholipases A_2 were determined at nanomolar or micromolar concentrations in the culture medium. A control substrate for phospholipases A_2 activity was established with the lecithin 1-octadecanoyl 2-[^{3}H] eicosatetraenoyl-sn-glycero-3-phosphocholine ([^{3}H] 20:4-GPC), a source of [^{3}H] arachidonic acid after cleavage at position 2.

Non-stimulated- or ionophore A23187-stimulated macrophages incorporated extensively the [^{14}C] phospholipid analogue added at 30–4000 nM. At 4000 nM which induced 50% inhibition of the phospholipase, 40% of the dose was found associated with the [^{14}C] phospholipids of 2×10^6 stimulated macrophages after 120 min incubation, while only low amounts of [^{14}C] non-phosphorous lipids were detected. It is concluded that the [^{14}C] phospholipid analogue was readily taken up by the macrophages with limited hydrolysis.

Keywords: Phospholipid analogue; Phospholipase A_2 inhibition; Macrophage; Arachidonic acid; Eicosanoid

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INTRODUCTION

In acute inflammation or in experimental cell activation, arachidonic acid (20:4) release must be lowered to prevent the generation of eicosanoids which are potent lipid mediators. This can be achieved after inhibition of macrophage, neutrophil or lymphocyte phospholipase A_2 which cleaves 1,2-diacyl and 1-alkyl (alk-1′-enyl) 2-acyl phospholipids with choline (GPC), ethanolamine (GPE) or inositol (GPI) at position 2 of the glycerol.

Phospholipid analogues which are added to the culture medium of macrophages and which are not hydrolysable at position 2 of the glycerol are inhibitors of phospholipase A_2. The structures 1-decyl 2-octyl-GPC[1] or 1-dodecyl 2-octanamido 2-deoxy-GPC[2] were found more effective in lowering arachidonic acid release, with an IC_{50} of 4000 nM, than 1,2-dihexadecyl-GPC[3,4] or 1-octyl 2-octylthio 2-deoxy-GPC[5] both with an IC_{50} of 10000 nM. But all molecules lowered also eicosanoid synthesis. These data suggest that a higher inhibitory effect on the enzyme results from a higher uptake in cells of phospholipid analogues with short fatty chains.

Rates of incorporation into cells of phospholipid analogues are lacking while they have been reported for diacyl-GPC,[1-5] lyso phospholipids and lyso-like phospholipids.[6-11] This study illustrates the uptake by macrophages of the [^{14}C] phospholipid analogue with 2 short fatty chains, 1-dodecyl 2-[1-^{14}C] octanamido-sn-2-deoxy glycero-3-phosphocholine: the radiolabelling at position 2 allowed monitoring of its resistance to phospholipase A_2 and determination of the amount which had been taken up by rat peritoneal macrophages after 120 min incubation. The 1-octadecanoyl 2-[^{3}H] eicosatetraenoyl-GPC, ([^{3}H] 20:4-GPC) added into the culture medium represented a control for phospholipase A_2 activity since this lecithin is a substrate of the enzyme and source of [^{3}H] 20:4.

MATERIALS AND METHODS

Materials

Ionophore A23187, standard lipids, 1-octadecanoyl 2-eicosatetraenoyl-sn-glycero-3-phosphocholine (20:4-GPC) and other biochemicals were obtained from Sigma, USA. All chemicals and solvents were provided from Aldrich and Merck, D. The I.R. spectra were obtained using a Bomen MB-100. Nuclear magnetic resonance (^{1}HNMR) spectra were recorded on a Bruker AC 250 spectrophotometer. High resolution mass spectra were

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recorded on a HP 5889A quadripolar. Analytical thin layer chromatography (TLC) were performed on Merck 60 and 60F-254 silica gel plates. Preparative column chromatography was performed using Merck silica gel 60 (230–400 mesh).

The 1-octadecanoyl 2-[5,6,8,9,11,12,14,15,^{3}H] eicosatetraenoyl-sn-glycero-3-phosphocholine ([^{3}H] 20 : 4-GPC) of specific radioactivity: 5900 GBq/mmol with radiochemical purity of 98%, as measured on a TLC plate, was purchased from Amersham International, U.K. The [1-^{14}C] octanoic acid (specific radioactivity: 2.22 GBq/mmol) was from New England Nuclear, Boston, MA.

Synthesis of the [^{14}C] Phospholipid Analogue 1-Dodecyl-2-[1-^{14}C] Octanamido-sn-2-Deoxy Glycero-3-Phosphocholine and of the Non-radioactive Compound (Figure 1)

The D-serine amino group was protected with tert-butoxycarbonyl (BOC). The hydroxyl group was alkylated with dodecyl bromide in presence of NaH in dimethylformamide (DMF).[12] The acid group was converted to the corresponding caesium salt with aqueous caesium carbonate and then, esterified with benzyl bromide in DMF at 65°C.[13] The ester group was reduced with $LiBH_4$ in ether and then converted to phosphocholine with 2-chloro-2-oxo-1,3,2-dioxo-phosphocholine (1.2 eq.) in benzene and anhydrous trimethylamine in acetonitrile at 65°C for 48 h. The amino group was deprotected with trifluoroacetic acid (TFA) in CH_2Cl_2 and converted to an amide group with [1-^{14}C] octanoic acid (or octanoic acid) in the presence of dicyclohexylcarbodiimide and purified by thin layer chromatography on silica gel plates using the mobile phase chloroform/methanol/water (65 : 25 : 4 v/v). The radiochemical purity of the compound was 99.7%, and its specific radioactivity was 136 MBq/mmol.

Cell Isolations and Cultures

Rat peritoneal macrophages were prepared essentially as described previously.[14] Briefly, the peritoneal cavity from male Wistar A.F. rats (250–300 g) was washed with 50 ml sterile saline buffer. Washes were then performed by centrifugation (350 × *g*) for 10 min. The cell pellets were suspended in Dulbecco's phosphate buffer, pH 7.2 containing glucose (1 g/l) and after gentle agitation, aliquots of buffer containing the cells, were distributed in glass tubes. One hour incubation, at 37°C, was required to obtain adherent macrophages. Non-adherent neutrophils and lymphocytes

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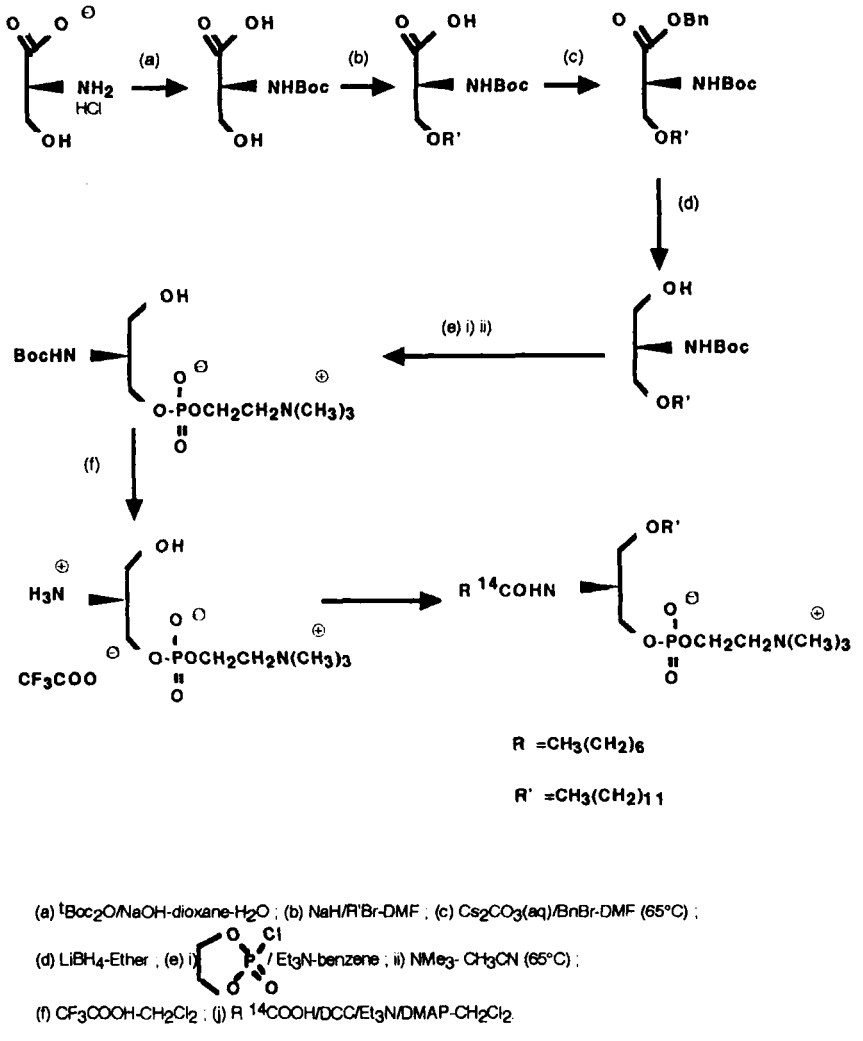

FIGURE 1 Chemical synthesis of the phospholipid analogue 1-dodecyl-2-[1-^{14}C] octanamido-sn-2-deoxy-glycero-3-phosphocholine.

were removed by 3 washes with phosphate buffer. Cell viability after 120 min of incubation was >95%, as observed by the trypan blue dye exclusion technique.

When [^{3}H] 20:4-GPC was added at 0.1 nM, the initial specific radioactivity was 5900 GBq/mmol. At other concentrations, the specific radioactivities were: 59 GBq/mmol (10 nM), 9.36 GBq/mmol (200 nM) and 1.77 GBq/mmol (1000 nM).

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[^{14}C] phospholipid analogue was always added to the culture medium at a specific radioactivity 136 MBq/mmol. To allow for the accurate detection of radioactive lipids in macrophages and extracellular fluids, the radioactive analogue was added to the medium at a concentration of, at least, 30 nM.

For experiments when [^{3}H] and [^{14}C] phospholipids were both added to the medium, the [^{3}H]/[^{14}C] ratio was never higher than 13 or lower than 1.

Non-stimulated- or stimulated macrophages were incubated in Dulbecco's phosphate buffer (culture medium) for 120 min. They were stimulated with ionophore A23187 at 10000 nM in the last 30 min. After incubations, extractions of total lipids were performed upon extracellular fluids and non-washed adherent cells or upon adherent cells washed 3 times with 20:4-GPC at 5000 nM or with non-labelled phospholipid analogue at 5000 nM or with both phospholipids at 5000 nM each. The third wash contained only 0.1–0.5% of the radioactivity which was not incorporated in macrophages. Total lipids were then extracted.

Lipid Analysis

Lipids from cells and extracellular fluids were extracted with chloroform and methanol.[15] Before extraction, the extracellular fluids were acidified at pH 4 with diluted formic acid. Aliquots of the chloroformic phase were counted for radioactivity or applied on TLC plates. The lipid types from radiolabelled lipid samples were separated in toluene/dioxan/formic acid/acetic acid (85:15:0.5:0.5 v/v). Phospholipids remained at the origin, while mono- and diglycerides run from Rf = 0.15–0.50, fatty acids at 0.80 and triglycerides at 0.90. Lipids from extracellular fluids were phospholipids, fatty acids and eicosanoids. Leukotrienes and prostaglandins were not separated and were at Rf = 0.05–0.60. The triglycerides were in traces. In addition, standard non-labelled lipids were applied on TLC plates (1,2,3-trioctadecenoyl glycerol 1,2-dioctadecenoyl glycerol, 1-octadecenoyl glycerol, octadecenoic acid, prostaglandin E_2, leukotriene B_4, 1-octadecanoyl 2-eicosatetraenoyl-GPC). However the [^{14}C] lipids which appeared after [^{14}C] phospholipid analogue degradation were not identified and migrated at Rf values corresponding to non-phosphorous lipids. Lipids were visualized with iodine vapour which was rapidly removed by blowing. Spots containing radioactive lipid classes from TLC plates and aliquots of the chloroform phases containing radioactive lipids were introduced into flasks. After evaporation of the solvents, 4 ml scintillation liquid and 0.4 ml distilled water were added. Radioactivity was measured in a LKB

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1209 scintillation spectrophotometer equipped with external standardization for the dual label counting method.

RESULTS

Uptakes of [^{3}H] 20:4-GPC or [^{14}C] Phospholipid Analogue by Non-stimulated Macrophages (Figures 2 and 3)

Uptakes were measured as total radioactive lipids recovered from macrophages.

Within 5 min of incubation, the [^{3}H] 20:4-GPC (Figure 2) was bound to non-washed cells, particularly at the lowest concentration 0.1 nM, to the extent of 28% of the dose. At 120 min with 200 and 1000 nM, the values reached 40–48%. Three washes with 20:4-GPC at 5000 nM in buffer removed [^{3}H] 20:4-GPC which was not incorporated in cells. Whatever the concentrations used, the percentage of [^{3}H] lipids which were not incorporated into cells was similar and increased from 6–8% at 5 min, to 11–15% at 30 min, and around 20–24% at 120 min.

The [^{14}C] phospholipid analogue (Figure 3) was rapidly and extensively bound to non-washed cells. After 120 min and for all concentrations used, the values were 48–54% of the dose. In cells which were washed 3 times with the non-labelled analogue at 5000 nM, the uptakes were similar whatever the initial concentration of the [^{14}C] phospholipid analogue in culture medium and increased from 5% at 5 min, to 22–26% at 30 min and 28–34% at 120 min. All values were higher than those found with [^{3}H] 20:4-GPC.

Simultaneous Uptakes of [^{3}H] 20:4-GPC and [^{14}C] Phospholipid Analogue by Non-stimulated Macrophages (Figure 4)

Uptakes were measured as total radioactive lipids recovered from macrophages. After incubations, the cells were washed 3 times with non-labelled phospholipids (each at 5000 nM).

Each radioactive phospholipid was present at 1000 nM in the culture medium. Irrespective of the time of incubation, about twice as much [^{14}C] lipids than [^{3}H] lipids were found in the cells: at 120 min, this was, respectively, 22% and 12% of the dose.

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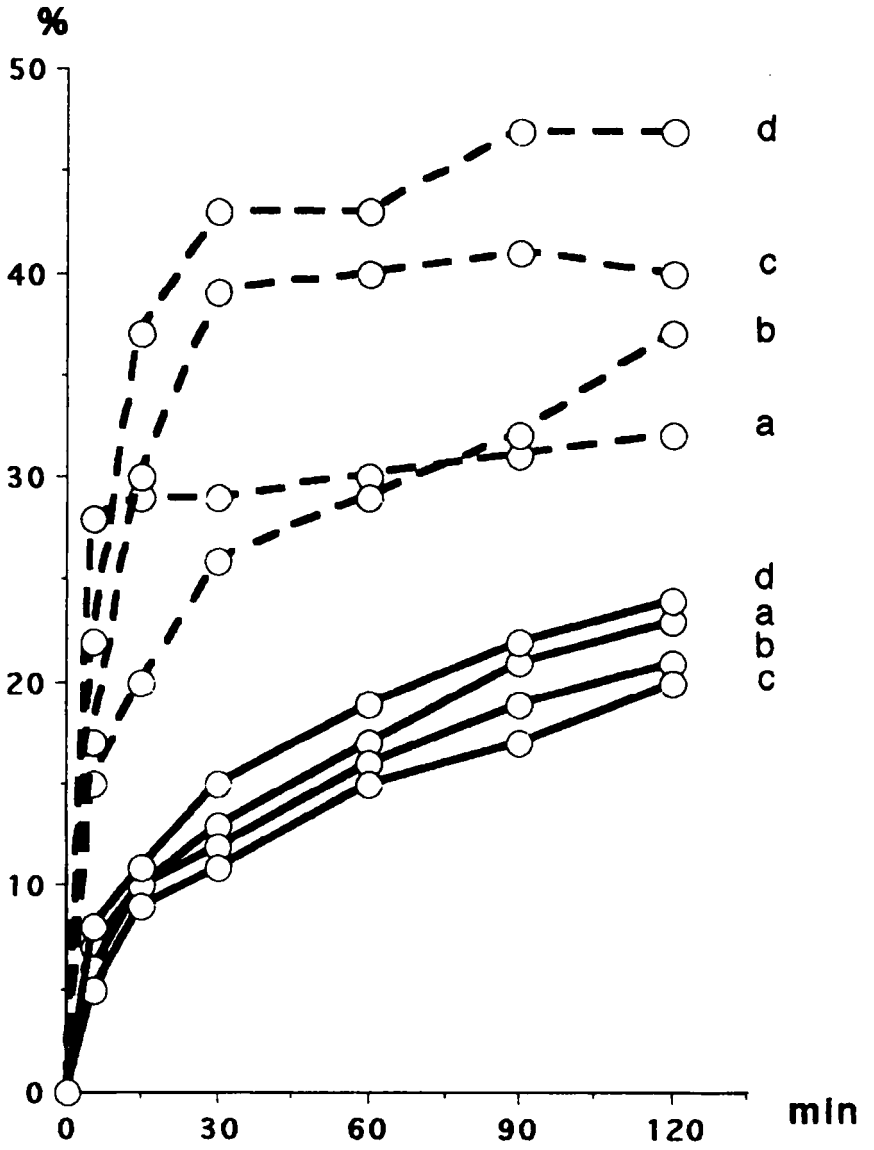

FIGURE 2 [^{3}H] lipids (% of the dose) extracted from 1×10^6 non-stimulated macrophages, according to time of incubation and concentrations in culture medium of 1-octadecanoyl-2-[^{3}H] eicosatetraenoyl-sn-glycero-3-phosphocholine ([^{3}H] 20:4-GPC). After incubations, the extracellular fluids were discarded. The cells were not washed (○---○) or washed 3 times with 20:4-GPC at 5000 nM in buffer (○—○). Lipids were then extracted. a, b, c, d are the concentrations 0.1, 10, 200 and 1000 nM of [^{3}H] 20:4-GPC in culture medium. Values are means from 2 experiments with extreme values at about 4% around the mean.

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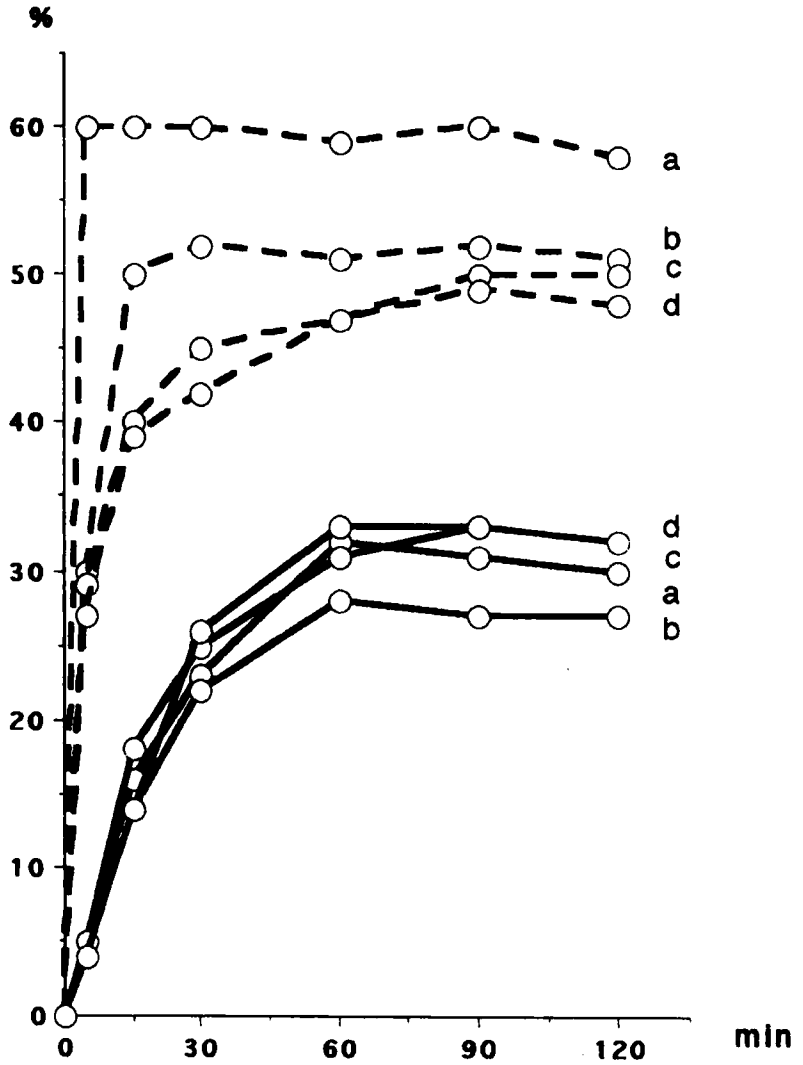

FIGURE 3 [^{14}C] lipids (% of the dose) extracted from 1×10^6 non-stimulated macrophages, according to time of incubation and concentration in culture medium of 1-dodecyl-2-[1-^{14}C] octanamido-sn-2-deoxy-glycero-3-phosphocholine ([^{14}C] phospholipid analogue). After incubations, the extracellular fluids were discarded. The cells were not washed (○---○) or washed 3 times with the non-radioactive phospholipid analogue at 5000 nM in buffer (○—○). Lipids were then extracted. a, b, c, d are concentrations 30, 250, 1500 and 4000 nM of [^{14}C] phospholipid analogue in the culture medium. Values are means from 2 experiments with extreme values at about 4% around the mean.

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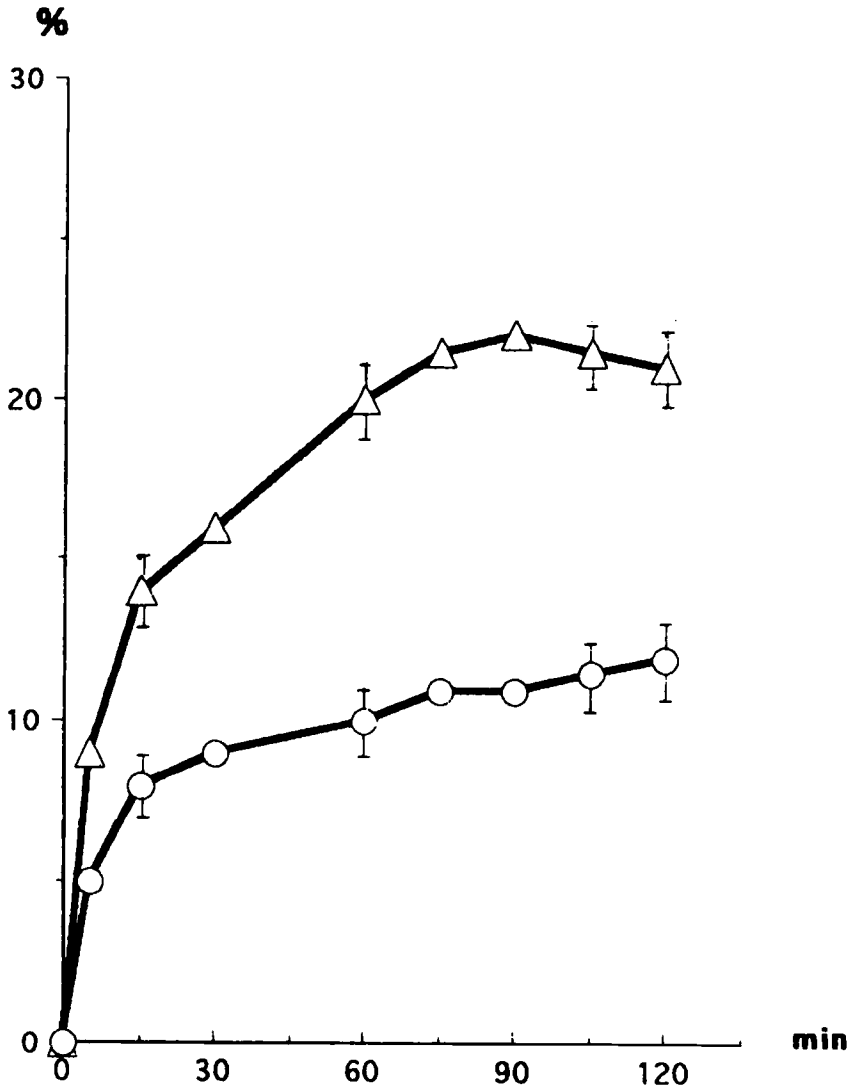

FIGURE 4 [^{3}H] and [^{14}C] lipids (% of the dose) extracted from 10^6 non-stimulated macrophages which received in culture medium, [^{3}H] 20:4-GPC, and [^{14}C] phospholipid analogue, both at 1000 nM. After incubation, the extracellular fluids were discarded and the cells were washed 3 times with buffer containing non-radioactive molecules each at 5000 nM. Lipids were then extracted. [^{3}H] lipids (○—○); [^{14}C] lipids (△—△). Values are means from 2 experiments with extreme values at about 4% around the mean. For 3 experiments they are means ± SEM.

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TABLE I [^{3}H] and [^{14}C] lipids and distribution in lipid classes (%) in extracellular fluids and stimulated macrophages (2×10^6 cells) after incubation with 1-octadecanoyl-2-[^{3}H] eicosatetraenoyl-sn-glycero-3-phosphocholine ([^{3}H] 20 : 4-GPC) and 1-dodecyl-2-[1-^{14}C] octanamido-sn-2-deoxy-glycero-3-phosphocholine ([^{14}C] phospholipid analogue)

Radiolabelled molecules placed in culture medium			[^{3}H] *and* [^{14}C] *lipids after incubation*				
	Concentrations (nM)	*Masses* (pmol)	*Total lipids** (% *of the dose*)		*Distribution in lipids classes* (%)†		
			Extra cellular fluids	*Macrophages*	*Extra cellular fluids*		*Macrophages*
[^{3}H] 20 : 4-GPC	0.1	0.2	66.5 ± 3.9	33.5 ± 1.8	PL	92.5	92.5
					NPL	7.5	7.5
[^{14}C] phospholipid analogue	1000	2000	49.3 ± 2.1	50.7 ± 2.3	PL	98.0	97.5
					NPL	2.0	2.5
[^{3}H] 20 : 4-GPC	0.1	0.2	59.0 ± 3.2	41.0 ± 2.6	PL	90.0	96.4
					NPL	10.0	3.6
[^{14}C] phospholipid analogue	4000	8000	60.0 ± 2.5	40.0 ± 1.9	PL	98.9	98.7
					NPL	1.1	1.3
[^{3}H] 20 : 4-GPC	1000	2000	89.5 ± 4.7	10.5 ± 0.9	PL	95.0	98.0
					NPL	5.0	2.0
[^{14}C] phospholipid analogue	250	500	71.9 ± 3.3	27.9 ± 1.2	PL	95.0	96.3
					NPL	5.0	3.7
[^{3}H] 20 : 4-GPC	1000	2000	85.2 ± 4.5	14.8 ± 0.9	PL	95.7	98.5
					NPL	4.3	1.5
[^{14}C] phospholipid analogue	1000	2000	52.2 ± 2.2	47.8 ± 2.3	PL	99.0	97.7
					NPL	1.0	2.3

†PL: Phospholipids. NPL: Non-Phosphorous Lipids. *Total lipids (% of the dose) are means ± SEM (3 separate experiments).

Simultaneous Uptakes of [^{3}H] 20 : 4-GPC and [^{14}C] Phospholipid Analogue by Ionophore A23187 Stimulated Macrophages (Table I)

After incubations, the cells were washed 3 times with non-labelled phospholipids (each at 5000 nM).

[^{3}H] 20 : 4-GPC at 0.1 nM and [^{14}C] Phospholipid Analogue at 1000 nM in Culture Medium

The macrophages took up 33.5% of 0.2 pmol of [^{3}H] 20 : 4-GPC and 50.7% of 2000 pmol of [^{14}C] phospholipid analogue.

[^{3}H] 20 : 4-GPC at 0.1 nM and [^{14}C] Phospholipid Analogue at 4000 nM in Culture Medium

The macrophages took up 41% of 0.2 pmol of [^{3}H] 20 : 4-GPC and 40% of 8000 pmol of [^{14}C] phospholipid analogue. At this concentration, the phospholipid analogue is known to inhibit phospholipase A_2 by 50%.

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In both experiments, the [^{3}H] and [^{14}C] lipids recovered on the TLC plates were largely PL (92.5–98.5% of the total radioactive lipids).

[^3H] 20:4-GPC at 1000 nM and [^{14}C] Phospholipid Analogue at 250 nM in Culture Medium

10.5% of 2000 pmol of [^{3}H] 20:4-GPC but 27.9% of 500 pmol of [^{14}C] phospholipid analogue were found in the cells.

[^3H] 20:4-GPC at 1000 nM and [^{14}C] Phospholipid Analogue at 1000 nM in Culture Medium

14.8% of 2000 pmol of [^{3}H] 20:4-GPC but 47.8% of 2000 pmol of [^{14}C] phospholipid analogue were found in the cells.

In both experiments, [^{3}H] and [^{14}C] lipids recovered on TLC plates were largely PL (96.3–98.5% of the total radioactive lipids).

In extracellular fluids, the rate of hydrolysis for [^{14}C] phospholipid analogue was lower than that observed for [^{3}H] 20:4-GPC.

DISCUSSION

Stimulation of macrophages is currently obtained after 5–30 min of incubation with ionophore A23187 at 1–10 μM.[1–5,16–20] Macrophage phospholipids are often labelled with [^{3}H] or [^{14}C] 20:4 (free form) used at picomolar or nanomolar concentration in culture medium in accord with routine experimental procedures.[4] But as described briefly in this study and detailed in other works,[1,2,5] the lecithin [^{3}H] 20:4-GPC, when added at 0.1 nM to the culture medium, was incorporated into macrophage phospholipids and represented an additional physiologic substrate for phospholipases. It may have been a substrate of secreted phospholipase A_2 associated with lysosomes, which is known to become active in stimulated macrophages. It may also have been a substrate of intracellular phospholipase A_2 which is active, after phosphorylation, in stimulated macrophages. However, these experiments were unable to evaluate the contribution of each enzyme which splits the [^{3}H] 20:4-GPC. The secreted enzyme acts on exogenous phospholipids which are organized above their critical micellar concentration. In these experiments, its contribution was uncertain because the substrate was never above a concentration of 1000 nM which is a low concentration to obtain micellar phospholipids. We have reported that in the absence of phospholipid analogue, the substrate was split at position 2 of the glycerol backbone with formation of [^{3}H] fatty acids in cells and [^{3}H] fatty acids and [^{3}H] eicosanoids in extracellular fluids, with hydrolysis

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corresponding to 15–20% of the dose.[1–3,5] But as seen in Table I, in the presence of the [^{14}C] phospholipid analogue at 4000 nM (which was the IC_{50} reported), the hydrolysis of the tritiated substrate (added at 0.1 nM to the culture medium) reached only 4% of the lipids in macrophages. The substrate [^{3}H] 20:4-GPC was in addition loaded in the culture medium at a concentration of 1000 nM which is considerably higher than 0.1 nM, in order to compare its uptake with values found with the [^{14}C] phospholipid analogue used at 1000 or 4000 nM. All the results in Figure 4 and Table I showed that added at 1000 nM each to the culture medium, the [^{14}C] phospholipid analogue was incorporated in higher amounts than the [^{3}H] 20:4-GPC.

The [^{14}C] phospholipid analogue was chosen among various structures for its potent inhibitory effect upon phospholipase A_2 in ionophore A23187-stimulated macrophages with an IC_{50} value of 4000 nM,[5] though other inhibitors with alkyl or thio bond used here were also efficient inhibitors.[1,5] But the common feature of these structures was that short fatty chains linked to the glycerophosphate backbone produced higher inhibitory effects than a phospholipid analogue with long fatty chains.[3] All the analogues synthetized here were probably recognized by phospholipase A_2 since they possessed 2 fatty chains of at least 7 or 8 carbons linked to position 1 and 2 of the glycerophosphate backbone.[21] Phospholipid analogues with long or short fatty chains linked by alkyl, thio or amido bonds to position 2 of the glycerol or equivalent backbone have also been studied for their inhibitory effects upon pure snake venom or pancreatic juice phospholipases A_2.[22–24] In conclusion, the [^{14}C] phospholipid analogue used in this study, with its amido bond at position 2 of the glycerophosphate backbone was one of the representative phospholipid analogues which developed inhibitory effects on phospholipases.

The [^{14}C] phospholipid analogue was not entirely protected against hydrolysis. Low amounts of [^{14}C] non-phosphorous lipids appeared in macrophages with 1–3.7% of the lipids (Table I). The [^{14}C] non-phosphorous lipids were not identified. They probably appeared after the action of phospholipases, though the phospholipid analogue was not a substrate for these enzymes. However, once incorporated into human promyelocytic and myelocytic leukemia cells, the lyso like phospholipid methyl PAF acether 1-[^{3}H] octadecyl 2-methyl-GPC, which resembles a phospholipid analogue, was slightly cleaved by phospholipase C: the diglyceride 1-[^{3}H] octadecyl 2-methyl glycerol has been isolated.[25] The etherases which are always active in non-transformed cells may have cleaved the alkyl bond at position 1 of the [^{14}C] phospholipid analogue with formation

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of dodecanol and 1-lyso 2-[^{14}C] octanamido 2-deoxy-GPC. This lyso compound would probably be rapidly esterified with a long chain fatty acid and would form the new [^{14}C] phospholipid analogue 1-acyl 2-[^{14}C] octanamido 2-deoxy-GPC; identification of this compound was not pursued.

At the IC_{50} which corresponded to 4000 nM in culture medium, 3200 picomoles of the [^{14}C] phospholipid analogue, which represented 40% of 8000 picomoles, were incorporated into 2×10^6 macrophages after 120 min of incubation, as calculated from the results shown in Table I. This value is near the amount of methyl PAF acether 1-octadecyl 2-methyl GP [^{14}C] choline which was incorporated into 1×10^6 RAJi or L1210 cells after 60 min of incubation at 5000 nM in culture medium.[26] Other phospholipids with 2 carbons at position 2 of the glycerol are also known to be rapidly incorporated into macrophages, leukocytes leukemia cells or enterocytes: i.e. 1-octadecyl 2-acetyl-GPC (PAF acether),[7,9,10] 1-hexadecylmercapto 2-methoxymethyl-GPC (Ilmofosine),[6] and 1-octadecyl 2-acetyl-glycerophosphate.[7] Considering that in an acute inflammation state, the capacity of a phospholipid analogue to lower the release of 20:4 from macrophages is of physiopathological relevance, these experiments give support to an important incorporation (40% of the dose) of the [^{14}C] phospholipid analogue at the concentration 4000 nM in culture medium where it achieved 50% inhibition of phospholipase A_2. Furthermore, it was relatively stable to phospholipases.

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