Acyl-chain selectivity of the 85 kDa phospholipase A₂ and of the release process in intact macrophages

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The selectivity of the intracellular 85 kDa phospholipase A₂ (PLA₂-85) towards fatty acids closely related to arachidonic acid has been investigated, using purified PLA₂-85 from J774 cells and mixed phospholipids, dually acyl-chain-labelled in the *sn*-2 position. In parallel experiments, we assessed the acyl-chain selectivity of the release process in intact, dually labelled, peritoneal mouse macrophages responding to either calcium ionophore or zymosan beads in the presence of indomethacin and BSA. The results obtained in the two systems were very

similar, which supports previous evidence that PLA₂-85 is responsible for stimulus-induced release of eicosanoid precursor in mouse macrophages. In the *in vitro* system, PLA₂-85 was found to exhibit a moderate selectivity towards C₂₀ acyl chains differing in double-bond structure, while the sensitivity to acyl-chain length was more pronounced. Together with previous data, these results demonstrate a striking preference for C₂₀ over either C₁₈ or C₂₂ unsaturated acyl chains.

INTRODUCTION

Receptor interactions leading to the formation of prostaglandins and other eicosanoids in macrophages are initiated by the release of precursor fatty acid from membrane phospholipids. 5,8,11,14-Eicosatetraenoic acid (C_{20:4(n-6)}; arachidonic acid) is the prevailing polyunsaturated fatty acid in most of the phospholipids from which eicosanoid precursor is mobilized. However, ethanolamine phospholipid is generally also very rich in docosahexaenoic acid (C_{22:6}). In addition, dietary changes, such as *cis,cis*-9,12-octadecadienoic acid (linoleic acid) deficiency and high intake of fish oils, may cause changes in the content of C_{20:4(n-6)} and other polyunsaturated fatty acids in macrophage phospholipids [1–3].

There is now considerable circumstantial evidence that an intracellular 85 kDa phospholipase A₂ (PLA₂-85) [4–8], also referred to as cytosolic PLA₂ or cPLA₂, is responsible for the mobilization of C_{20:4(n-6)} in macrophages. Regulatory roles for changes in cytosolic [Ca²⁺] and in degree of phosphorylation of PLA₂-85 have been documented in intact cells and been corroborated using purified enzyme [4–13].

It has been demonstrated previously that PLA₂-85 exhibits little specificity with regard to the polar head group of the major phospholipids phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol; nor does it appear to discriminate to any major extent between 1-*O*-alkyl 2-arachidonoyl and 1-acyl 2-arachidonoyl phosphatidylcholine [8,9,13,14]. On the other hand, 2-palmitoyl, 2-oleoyl and 2-linoleoyl phosphatidylcholine are hydrolysed at a much lower rate than their 2-arachidonoyl counterpart [5,6,8,9,13–17], indicating that PLA₂-85 is sensitive to the structure of the fatty acid in position 2 of its substrate phospholipid. However, to what extent this reflects sensitivity to differences in chain length and/or degree of unsaturation has only recently been addressed [16]. We have therefore compared the ability of PLA₂-85 to release C_{20:4(n-6)} and some closely related, polyunsaturated, C₂₀ and C₂₂ fatty acids. In addition, we have compared the acyl-chain selectivity of PLA₂-85 with that

for the release in intact macrophages, responding to either calcium ionophore or zymosan beads.

EXPERIMENTAL

Mouse peritoneal macrophages were isolated as described previously [18], and cultured in 6- or 12-well tissue-culture dishes (Costar). Cells were labelled for 20 h with [³H]C_{20:4(n-6)} (0.2–0.5 μCi; specific radioactivity 80–130 Ci/mmol; Amersham International) and either [1-¹⁴C]-labelled C_{20:3(n-9)}, C_{20:3(n-6)}, C_{20:5} or C_{22:6} (0.2 μCi; specific radioactivity 55 Ci/mol; American Radiolabelled Chemicals, St. Louis, MO, U.S.A.). At that time, 38–44% of each of the radiolabelled fatty acids added was esterified in macrophage lipids. In some experiments, the labelling time with [¹⁴C]C_{20:5} was shortened to 30–45 min, or varied as indicated.

For experiments with intact cells, macrophage cultures, labelled as described above, were equilibrated in fresh Medium 199 containing fatty-acid-depleted BSA (0.1%, w/v) and indomethacin (1 μM) and were then exposed to calcium ionophore (A23187; 1 μM) for 30 or 60 min, or to zymosan beads (0.2 mg/ml) for 30 min. The culture medium was collected, subjected to centrifugation (3000 rev./min for 5 min) and the ³H- and ¹⁴C-labelled fatty acids released were then determined in the supernatant. Radiolabelled fatty acids which remained esterified in cellular lipids were determined after solubilization of the cell monolayer in Triton X-100 (0.1%, w/v). Some control cultures were subjected instead to lipid extraction [18] and either fractionation on a column of silicic acid into neutral lipids (chloroform eluate) and phospholipids (methanol eluate; for use as substrate in the phospholipase assay; see below), or for t.l.c. [18], to determine the distribution of the radiolabels among the various phospholipids.

For experiments with purified PLA₂-85 from J774 cells [8], phospholipids prepared from dually labelled macrophage cultures (see above) were used as substrates in the form of sonicated vesicles. The assay mixture in addition contained (total volume

Abbreviations used: PLA₂-85, 85 kDa phospholipase A₂; C_{20:3(n-9)}, eicosa(5,8,11)trienoic acid; C_{20:3(n-6)}, eicosa(8,11,14)trienoic acid; C_{20:4(n-6)}, eicosa(5,8,11,14)tetraenoic acid; C_{20:5}, eicosapentaenoic acid; C_{22:5}, docosapentaenoic acid; C_{22:6}, docosahexaenoic acid.

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0.5 ml): KCl (80 mM), Hepes (10 mM, pH 7.4), EDTA (1 mM), CaCl_2 (290 μM free Ca^{2+}) and fatty-acid-depleted BSA (100 μg). After incubation for 5–30 min at 37 °C the reaction was stopped and the liberated fatty acids were separated from remaining substrate on a column of silicic acid, as previously described [5].

The interconversions undergone by the radiolabelled fatty acids found esterified in macrophage phospholipids were monitored by isocratic, reverse-phase h.p.l.c. (eluent: acetonitrile/ H_2O ; 9:1, v/v) and on-line radioactivity determination (System Gold, Beckman Instruments), after transesterification in 2% (v/v) of H_2SO_4 in methanol/toluene (1:1, v/v), essentially as described [19,20].

RESULTS AND DISCUSSION

Radiolabelled $\text{C}_{20:4(n-6)}$, $\text{C}_{20:3(n-9)}$, $\text{C}_{20:3(n-6)}$, $\text{C}_{20:5}$ and $\text{C}_{22:6}$ were all incorporated to a similar extent into macrophage lipids (see the Experimental section). Neither esterified $\text{C}_{20:3(n-6)}$, $\text{C}_{20:3(n-9)}$ nor $\text{C}_{22:6}$ were found to have undergone any significant chain elongation or desaturation during the 20-h labelling period, and only a minor part (< 15%) of the $\text{C}_{20:4(n-6)}$ had been elongated to docosatetraenoic acid. In contrast, $\text{C}_{20:5}$ was almost quantitatively elongated to $\text{C}_{22:5}$ at this time (Figure 1). This agrees with a previous report [19] and enabled us to evaluate the ability of PLA_2 -85 to release this fatty acid also. After short-term (30–45 min) labelling with $\text{C}_{20:5}$, up to 85% of the radiolabelled, phospholipid-bound fatty acid was still $\text{C}_{20:5}$ (Figure 1). Such preparations were used to evaluate the selectivity of PLA_2 -85 towards this fatty acid. Each of the various fatty acids became distributed somewhat differently among the major macrophage phospholipids (Table 1). Among more notable differences were the low incorporation of $\text{C}_{22:5}$, and particularly $\text{C}_{22:6}$, into phosphatidylinositol and of the two $\text{C}_{20:3}$ fatty acids into ethanolamine phospholipid. The enrichment of $\text{C}_{22:6}$ in ethanolamine phospholipid compared with other phospholipids was

Figure 1 Time course for the elongation of $\text{C}_{20:5}$ into $\text{C}_{22:5}$ in mouse macrophages

Primary macrophage cultures were exposed to $[^{14}\text{C}]\text{C}_{20:5}$ (0.2 μCi) for the time indicated. Fatty acids derived from macrophage phospholipids were analysed as described in the Experimental section. The percentage of radiolabel recovered as $\text{C}_{22:5}$ is shown as a function of labelling time. Each type of open symbol denotes a separate experiment, while the filled symbol is representative of several separate experiments.

Table 1 Distribution of esterified, radiolabelled $\text{C}_{20:4(n-6)}$, $\text{C}_{20:3(n-9)}$, $\text{C}_{20:3(n-6)}$, $\text{C}_{20:5}$, $\text{C}_{22:5}$ and $\text{C}_{22:6}$ among major macrophage phospholipids

Data are means for two or three separate experiments with very similar results. The labelling time was 20 h, except for $\text{C}_{20:5}$, where cells were labelled for 30 min. Data for $\text{C}_{22:5}$ were obtained from cultures labelled for 20 h with $[^{14}\text{C}]\text{C}_{20:5}$.

Lipid	Content (% of phospholipid radioactivity)					
	$\text{C}_{20:3(n-6)}$	$\text{C}_{20:3(n-9)}$	$\text{C}_{20:4(n-6)}$	$\text{C}_{20:5}$	$\text{C}_{22:5}$	$\text{C}_{22:6}$
Ethanolamine phospholipid	16.5	18.0	36.4	6.3	32.7	50.0
Phosphatidylcholine	51.6	50.0	32.6	74.3	37.4	27.2
Phosphatidylinositol	9.6	14.0	14.3	15.5	6.7	2.2
Phosphatidylserine	15.8	10.0	7.4	1.1	12.2	8.0

expected, and the virtually exclusive recovery of $\text{C}_{20:5}$ in phosphatidylcholine and phosphatidylinositol was probably due to the short labelling time used here, as a rapid initial labelling of these two phospholipids followed by a slower transfer into ethanolamine phospholipid is known to occur for $\text{C}_{20:4(n-6)}$ ([21] and references therein). The differences in phospholipid distribution of the various fatty acids would not be expected to have any major impact on the results reported in this paper, as PLA_2 -85 exhibits little selectivity towards the relevant phospholipid head groups in mixed vesicles. Furthermore, previous studies on intact macrophages have shown a very similar relative release of radiolabelled $\text{C}_{20:4(n-6)}$ from ethanolamine, choline and inositol phospholipids when the cells were stimulated with either calcium ionophore [21] or zymosan [18].

Purified PLA_2 -85 was incubated with phospholipid vesicles containing esterified ^3H -labelled $\text{C}_{20:4(n-6)}$ and either ^{14}C -labelled $\text{C}_{20:3(n-9)}$, $\text{C}_{20:3(n-6)}$, $\text{C}_{20:5}$, $\text{C}_{22:5}$ or $\text{C}_{22:6}$. The results from those experiments, together with data on the liberation of phospholipid-bound oleic and linoleic acid [8], are shown in Figure 2. The preference of PLA_2 -85 for esterified $\text{C}_{20:4(n-6)}$ over the tri- and penta-enoic C_{20} fatty acids was rather modest (1.3–1.9-fold), whereas the preference over the C_{22} fatty acids was surprisingly large (≥ 5 -fold) and more pronounced than that over oleic and linoleic acid. Our data for $\text{C}_{20:3(n-9)}$ agree exactly with those reported recently for human PLA_2 -85 [16], while the reported relative liberation of $\text{C}_{20:3(n-6)}$ is much lower than in our system. As noted earlier, $\text{C}_{20:5}$ is readily converted via both the cyclooxygenase [22] and the 5-lipoxygenase [23] pathways, while the C_{22} fatty acids, notably $\text{C}_{22:6}$, are not metabolized via either of these pathways [24]. The efficient elongation of $\text{C}_{20:5}$ to $\text{C}_{22:5}$ in macrophages thus converts a fatty acid that is rather readily liberated by PLA_2 -85 and metabolized by the oxygenating pathways, into one that is only poorly liberated.

To assess the acyl-chain selectivity of the release process in intact cells, and the extent to which PLA_2 -85 would contribute to it, we first investigated the calcium-mediated mobilization of the corresponding polyunsaturated fatty acids in intact, dually radiolabelled macrophages in response to calcium ionophore. As a fraction of each fatty acid was esterified in neutral lipids, and therefore not available to PLA_2 -85 or other phospholipases, the data were corrected in order to express specifically the relative release from cellular phospholipids (Table 2). The selectivity for esterified $\text{C}_{20:4(n-6)}$ over other polyunsaturated fatty acids in intact cells was found to be very similar to that seen in the *in vitro* system, using purified PLA_2 -85 (Figure 2). Also when zymosan was used as the stimulus, the acyl-chain selectivity was quite

Table 2 Acyl-chain selectivity of the release process in intact macrophages

Dually acyl-chain-labelled, intact macrophages (see the Experimental section) were stimulated with either calcium ionophore (A23187; 1 μ M) or zymosan beads, in the presence of indomethacin (1 μ M) and BSA (0.1%, w/v). The stimulus-induced release of each ¹⁴C-labelled fatty acid [$C_{20:3(n-6)}$, $C_{20:3(n-9)}$ or $C_{22:6}$], relative to the release of [3 H] $C_{20:4(n-6)}$ was determined and is expressed as mean $\pm$ S.D. ($n = 3-16$). Data have been corrected for the presence of each fatty acid in neutral lipids and therefore represent release from cellular phospholipids.

Stimulus	Relative release (% of the release of $C_{20:4(n-6)}$)		
	$C_{20:3(n-6)}$	$C_{20:3(n-9)}$	$C_{22:6}$
A23187	52.0 $\pm$ 8.0	65.0 $\pm$ 2.5	13.1 $\pm$ 5.6
Zymosan	54.7 $\pm$ 1.1	59.9 $\pm$ 4.2	18.6 $\pm$ 1.8

Figure 2 Acyl-chain selectivity of PLA₂-85 assessed *in vitro*

The activity of purified PLA₂-85 from J774 cells [8] against phospholipid-bound polyunsaturated fatty acids was determined using sonicated vesicles of macrophage phospholipids, biosynthetically labelled with [3 H] $C_{20:4(n-6)}$ ($C_{20:4}$) and either [14 C] $C_{20:3(n-6)}$, [14 C] $C_{20:3(n-9)}$ (for 30 min or 20 h) or [14 C] $C_{22:6}$ in the standard assay (see the Experimental section [5]). The activity is expressed relative to that against arachidonoyl phospholipid ($C_{20:4}$). Data for $C_{22:5}$ were obtained using phospholipids from macrophages labelled for 20 h with [14 C] $C_{20:5}$. Each bar represents mean $\pm$ S.D. ($n = 8-13$). Data for oleic acid ($C_{18:1}$) and linoleic acid ($C_{18:2}$) are from [8].

similar (Table 2). It should be noted here that zymosan causes persistent activation of PLA₂-85 [11], most likely due to regulatory phosphorylation of the enzyme. The release of $C_{20:5}$ and $C_{22:5}$ was not assessed separately, but the composite, relative release of [14 C] $C_{20:5}$ and [14 C] $C_{22:5}$, after labelling with [3 H] $C_{20:4(n-6)}$ and [14 C] $C_{20:5}$ for 20 h, was determined in parallel, in a series of experiments using, on the one hand, intact macrophages and, on the other hand, purified PLA₂-85 and phospholipids from such cells. The relative release of 14 C in intact cells amounted to 29 $\pm$ 11 % of that of 3 H (mean $\pm$ S.D., $n = 15$) upon stimulation with ionophore A23187 and to 30.8 % (mean, $n = 2$) upon stimulation with zymosan. In the phospholipase assay, the release of 14 C amounted to 31 $\pm$ 5 % ($n = 13$) of 3 H release. These results and those of Figure 2 and Table 2 lend strong support to the view that PLA₂-85 accounts for the

stimulus-induced mobilization of polyunsaturated fatty acids in macrophages.

As PLA₂-85 shows little selectivity towards phospholipid polar head groups, or the type of linkage at the *sn*-1 position, the ability of the enzyme to clearly discriminate between C_{20} and C_{22} fatty acids is remarkable. Although we can only speculate as to its molecular basis, it appears likely that phospholipids containing a C_{20} fatty acid at the *sn*-2 position differ in conformation from those containing a C_{22} fatty acid in or around the ester bond cleaved by the enzyme.

The two experimental systems used in this study have one limitation in common; the abundance of the various molecular species of phospholipid was not easily varied experimentally and is likely to be lower for at least those containing $C_{20:3(n-9)}$, $C_{20:3(n-6)}$ and $C_{20:5}$, and possibly also $C_{22:5}$, compared with $C_{20:4(n-6)}$. However, previous experience with mixtures of oleoyl and arachidonoyl phosphatidylcholine (J. Wijkander and R. Sundler, unpublished work) has not provided any evidence that this would affect the apparent acyl-chain selectivity of PLA₂-85. It is therefore likely that the acyl-chain selectivity of PLA₂-85 and the relative abundance of the various *sn*-2 acyl chains in macrophage membranes accessible to the enzyme would be major determinants of the amount of each fatty acid mobilized. This would account for a number of previous observations, e.g. the relative formation of prostaglandins E₁ and E₂ [25], the relative mobilization of $C_{20:3(n-9)}$ and $C_{20:4(n-6)}$ [3], and the inhibitory effect of substitution with (*n*-3) fatty acids on leukotriene formation [26] despite the lack of a direct inhibitory effect of these fatty acids on the 5-lipoxygenase [24,27].

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