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Evidence for the regulation of guinea-pig heart microsomal phosphatidylcholine-hydrolysing phospholipase A_1 by guanosine 5′-[γ-thio]triphosphate

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We have recently characterized lysophospholipase A_2 activities in guinea-pig heart microsomes and postulated that these enzymes act sequentially with phospholipases A_1 to release fatty acids selectively from phosphatidylcholine (PC) and phosphatidylethanolamine, thus providing an alternative route to the phospholipase A_2 mode of release. In a further investigation of the postulated pathway, we have characterized the PC-hydrolysing phospholipase A_1 in guinea-pig heart microsomes. Our results show that the enzyme may have a preference for substrates with $C_{16:0}$ over $C_{18:0}$ at the *sn*-1 position. In addition, although the enzyme cleaves the *sn*-1 fatty acid, the rate of hydrolysis of PC substrates with $C_{16:0}$ at the *sn*-1 position was influenced by the nature of the fatty acid at the *sn*-2 position. The order of decreasing preference was $C_{18:2} > C_{20:4} = C_{18:1} > C_{16:0}$. The hydrolyses of the molecular species were differentially affected by heating at 60 °C. An investigation into the effect of nucleotides on the activity of the enzyme showed that guanosine 5′-[γ-thio]triphosphate (GTP[S]) inhibited the hydrolysis of PC by phospholipase A_1 activity, whereas GTP, guanosine 5′-[β-thio]diphosphate (GDP[S]), GDP, ATP and adenosine 5′-[γ-thio]triphosphate (ATP[S]) did not affect the activity. The inhibitory effect of GTP[S] on phospholipase A_1 activity was blocked by preincubation with GDP[S]. A differential effect of GTP[S] on the hydrolysis of different molecular species was also observed. Taken together, the results of this study suggest the presence of more than one phospholipase A_1 in the microsomes with different substrate specificities, which act sequentially with lysophospholipase A_2 to release linoleic or arachidonic acid selectively from PC under resting conditions. Upon stimulation and activation of the G-protein, the release of fatty acids would be inhibited.

INTRODUCTION

The selective release of fatty acids from phospholipids has been established as the rate-limiting step in the synthesis of eicosanoids [1]. It is generally accepted that direct release by phospholipase A_2 (PLA_2) is the major pathway for such release [1–3]. However, most of the methodology used to implicate the involvement of PLA_2 in releasing fatty acids from phospholipids does not exclude the involvement of other enzymes in the process [3]. One potential mechanism that has been suggested by us and other investigators is the liberation of arachidonic acid or other fatty acids at the C-2 position via the sequential action on the phospholipid by a phospholipase A_1 (PLA_1), followed by a lysophospholipase A_2 ($LPLA_2$ [2–5]). We have previously established the presence of two $LPLA_2$ activities in guinea-pig heart microsomes [4,5]. The characteristics of the enzymes led to suggestions they could work in concert with PLA_1 to release linoleic, arachidonic and possibly other polyunsaturated fatty acids from phosphatidylcholine (PC) and phosphatidylethanolamine, with the specificity of release being influenced by the selectivity of the PLA_1 for specific molecular species of the phospholipids [4,5]. The possible existence of PLA_1 with specificities for phospholipid molecular species was demonstrated with the purification of an enzyme from hamster heart cytosol with PLA_1 activity that showed a preference for PC molecules with $C_{20:4}$ at the C-2 position [6]. There is no report of a membrane-bound PLA_1 displaying a preference for specific molecular species of any phospholipid.

If the postulated $PLA_1/LPLA_2$ pathway is involved in the selective release of fatty acids, the activity of PLA_1 would be expected to be modulated by external stimuli acting on membrane receptors. There is much evidence in the literature supporting G-protein modulation of other phospholipases (A_2, C and D [7–11]); however, there is no report of modulation of PLA_1 activity by G-proteins which would imply a link to activated receptors. In the present study, we have investigated the specificity of PLA_1 activity in guinea-pig heart microsomes for defined PC species and also investigated the possible modulation of the activity by guanine nucleotides.

MATERIALS AND METHODS

Materials

1-Palmitoyl-2-[1-^{14}C]palmitoyl-GPC (GPC = *sn*-glycero-3-phosphocholine) (55 mCi/mmol), and 1-palmitoyl-2-[1-^{14}C]-arachidonoyl-GPC (50 mCi/mmol) were obtained from DuPont Canada (Mississauga, Ont., Canada). 1-Palmitoyl-2-[1-^{14}C]linoleoyl-GPC (54 mCi/mmol), 1-stearoyl-2-[1-^{14}C]arachidonoyl-GPC (56 mCi/mmol) and 1-palmitoyl-2-[1-^{14}C]oleoyl-GPC (54 mCi/mmol) were purchased from Amersham International. 1-Palmitoyl-2-linoleoyl-GPC, dipalmitoyl-GPC, 1-palmitoyl-2-oleoyl-GPC, sodium deoxycholate, Triton X-100, Triton QS-15, Tween-20 and sodium taurodeoxycholate were acquired from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 1-Palmitoyl-2-arachidonoyl-GPC and 1-stearoyl-2-arachidonoyl-GPC were obtained from Avanti Polar Lipids (Pelham, AL, U.S.A.). Guinea pigs were purchased from Charles River, St. Constant, Quebec, Canada. Pig liver lysophosphatidylcholine was obtained from Serdary Research Laboratories (London, Ont., Canada). Ecolite was purchased from ICN Biomedical (St. Laurent, Quebec,

Abbreviations used: (L)PLA, (lyso)phospholipase A; GPC, *sn*-glycero-3-phosphocholine; (L)PC, (lyso)phosphatidylcholine; GTP[S], guanosine 5′-[γ-thio]triphosphate; GDP[S], guanosine 5′-[β-thio]diphosphate; ATP[S], adenosine 5′-[γ-thio]triphosphate.

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Canada). All nucleotides were obtained from Boehringer Mannheim (Laval, Quebec, Canada). All other chemicals and solvents were of reagent grade and obtained from Baxter–Canlab (Winnipeg, Manitoba, Canada).

Preparation of subcellular fractions

For each experiment, membrane fractions were prepared from six to eight guinea-pig hearts by differential centrifugation as previously described [4]. The protein content of the heart microsomes was determined by the method of Lowry *et al.* [12].

Preparation of PC substrates

The specific PC substrates were prepared by addition of unlabelled and radiolabelled PC to a test tube to give the appropriate specific radioactivity (0.2–0.4 Ci/mol). Solvents were evaporated with N_2, followed by addition of a portion of sodium taurodeoxycholate (5%) to give a ratio of 0.01 g of detergent/0.5 μmol of PC. The volume of the solution was adjusted to 1 ml by addition of double-distilled water and the mixture was vortex-mixed for 30–60 s.

PLA_1 assays

PLA_1 was assayed by measuring the rate of formation of radiolabelled LPC from PC. The assay mixture contained 300 μM-PC (sp. radioactivity 0.2–0.4 Ci/mol), 100 μg of guinea-pig heart microsomal protein and 100 mM-Tris/HCl, pH 8.0, in a total volume of 500 μl. The reaction was initiated by addition of the PC substrate, and incubation was carried out at 37 °C for 10 min in a shaking water bath. The reaction was terminated by addition of 3 ml of chloroform/methanol (2:1, v/v) and 1 ml of 0.9% KCl. The tubes were vortex-mixed and centrifuged. The upper phase was removed, and reaction products in samples of the lower layer were separated by t.l.c. in chloroform/methanol/water/acetic acid (35:15:2:1, by vol.) as the development solvent. The bands were made visible with iodine, and radioactivity associated with the LPC band was determined by scintillation counting in a Beckman LS3801 scintillation counter with Ecolite scintillant.

Statistics

Statistical significance between groups was assessed by the unpaired *t* test.

RESULTS AND DISCUSSION

When PLA_1 activity was assayed in guinea-pig heart microsomes, very little activity (0–3 nmol/h per mg of protein) was obtained in the absence of detergent, irrespective of the PC substrate used. Taurodeoxycholate has been previously used for PLA_1 assays [13]; this detergent was therefore used to establish the optimum assay conditions with 1-$C_{16:0}$-2-$C_{18:2}$-GPC, 1-$C_{16:0}$-2-$C_{20:4}$-GPC and 1-$C_{16:0}$-2-$C_{16:0}$-GPC as substrates. An optimum pH of 8.0 was established for the hydrolysis of all substrates. The effect of increasing substrate concentration (0–400 μM) on activity revealed that there was no activity until a substrate concentration of 100 μM, a clear indication that the enzyme acts on micelles. The PLA_1 activity reached a peak at 300 μM and had declined by 35–45% at 350 μM substrate. The substrate concentration used in all subsequent experiments was 300 μM. The effects of other detergents (Triton X-100, Triton QS15, Tween 20 and deoxycholate) on the PLA_1 activity were examined. PLA_1 activity was only observed with deoxycholate. The substrate-concentration/activity profile with deoxycholate was similar to that of taurodeoxycholate; however, the specific activities were one-third of those observed with taurodeoxycholate. The final concentration of taurodeoxycholate used in the assays (10 mM) did not inhibit the hydrolysis of the substrates used in the studies. Ca^{2+} (100–500 μM) had no effect on the activity of PLA_1 (results not shown).

PLA_1 hydrolyses the *sn-1* fatty acid from phospholipids. To determine if the enzyme displayed any selectivity for the *sn*-1 fatty acid, we compared the relative hydrolysis of 1-$C_{16:0}$-2-$C_{20:4}$-GPC and 1-$C_{18:0}$-2-$C_{20:4}$-GPC at their optimum substrate concentrations by the guinea-pig heart microsomal PLA_1 activity. The results, which are displayed in Table 1, indicated there was significantly greater hydrolysis of 1-$C_{16:0}$-2-$C_{20:4}$-GPC than of 1-$C_{18:0}$-2-$C_{20:4}$-GPC. Although this suggests that the PC-hydrolysing PLA_1 activity may have a preference for substrates with $C_{16:0}$ at the C-1 position, studies with a larger number of molecular species are needed before a definitive conclusion can be made. We next compared the relative hydrolysis of PC molecules with $C_{16:0}$ at the C-1 position but different fatty acids at the C-2 position. The order of decreasing hydrolysis (Table 1) was $C_{18:2} > C_{20:4} = C_{18:1} > C_{16:0}$, thus revealing that the nature of the fatty acid at the C-2 position significantly affected the hydrolysis of the C-1 fatty acid. The relative rate of hydrolysis of 1-$C_{16:0}$-2-$C_{18:2}$-GPC and 1-$C_{16:0}$-2-$C_{20:4}$-GPC was the same when experiments were conducted with deoxycholate instead of taurodeoxycholate. However, the absolute values with deoxycholate were one-third of those with taurodeoxycholate. Thus the substrate specificity was not dependent on the nature of the detergent used in the assay. There are suggestions that the reported selectivity of phospholipases, particularly PLA_2 for $C_{20:4}$ molecular species of phospholipids may simply reflect the ease with which the enzyme penetrates different phospholipid micelles [14,15]. It is unlikely that a similar reason is responsible for the observed selectivity of PLA_1 in this study for the following reasons: (*a*) all the assays were conducted with mixed micelles of phospholipid and taurodeoxycholate; (*b*) the ease of penetration of micelles by the enzyme should favour the hydrolysis of $C_{20:4}$-containing species [14,15]; however, the rate of hydrolysis of 1-$C_{16:0}$-2-$C_{20:4}$-GPC was no different from 1-$C_{16:0}$-2-$C_{18:1}$-GPC and much less than the hydrolysis of 1-$C_{16:0}$-2-$C_{18:2}$-GPC. Furthermore, the rates of hydrolysis of the disaturated species 1-$C_{16:0}$-2-$C_{16:0}$-GPC and 1-$C_{18:0}$-2-$C_{20:4}$-GPC were similar. The PC-hydrolysing PLA_1 activity in guinea-pig heart microsomes therefore exhibits a preference for $C_{18:2}$ at the C-2 position and may have a preference for $C_{16:0}$ at the C-1 position. This selectivity of the PLA_1 for $C_{18:2}$-containing PC species is similar to that of the guinea-pig heart microsomal 1-lyso-2-acyl-GPC-hydrolysing lysophospholipase activity [4]. The hydrolysis of all the molecular species by a single PLA_1 would, on the basis of the specificity displayed by the PLA_1 and $LPLA_2$ suggest that the sequential action of the two enzymes would preferentially release linoleic acid from PC. Coincidentally, in the guinea-pig heart, most of the PC molecules have linoleic acid at the C-2 position [16].

If the postulated PLA_1/$LPLA_2$ pathway is involved in the selective release of fatty acids, the activity of PLA_1 would be expected to be modulated by external stimuli acting on membrane receptors. Since we are not aware of any PLA_1-activating agonists, we investigated the possibility of the receptor activation of PLA_1 being mediated by G-proteins. G-proteins are activated directly by non-hydrolysable analogues such as guanosine 5′-[γ-thio]triphosphate (GTP[S]), and therefore the response of effectors to these compounds is taken as evidence for G-protein mediation between receptors and the effector [7,17–20]. The hydrolysis of 1-$C_{16:0}$-2-$C_{18:2}$-GPC and 1-$C_{16:0}$-2-$C_{20:4}$-GPC by PLA_1 in guinea-pig heart microsomes was assayed in the presence of GTP[S] (0–1 mM). The results obtained are displayed in Table 2. Surprisingly, GTP[S] inhibited rather than activated the hydrolysis of both substrates. Hydrolysis of 1-$C_{16:0}$-2-$C_{20:4}$-GPC was inhibited by 50–60% by the GTP analogue, and inhibition

Table 1. Acyl specificity of guinea-pig heart microsomal PLA_1

The acyl specificity of PLA_1 was determined with different molecular species of PC. The substrates were presented as mixed micelles with taurodeoxycholate. The values represent means ± S.D. of four experiments each done in triplicate: * $P < 0.005$.

Substrate	Specific activity (nmol of LPC/h per mg of protein)	Activity relative to hydrolysis of 1-$C_{18:0}$-2-$C_{20:4}$ PC
1-$C_{18:0}$-2-$C_{20:4}$PC	24 ± 4	1.0
1-$C_{16:0}$-2-$C_{16:0}$ PC	26 ± 5	1.1
1-$C_{16:0}$-2-$C_{18:1}$ PC	32 ± 2*	1.3
1-$C_{16:0}$-2-$C_{20:4}$ PC	33 ± 5*	1.4
1-$C_{16:0}$-2-$C_{18:2}$ PC	50 ± 5*	2.1

Table 2. Effect of GTP[S] on guinea-pig heart microsomal PLA_1 activity

PLA_1 activity was assayed with GTP[S] (0–1 mM). The assays were performed as described in the Materials and methods section. The specific activities of PLA_1 with no addition were 60 ± 9 and 41 ± 3 nmol of LPC formed/h per mg of protein with 1-$C_{16:0}$-2-$C_{18:2}$-GPC and 1-$C_{16:0}$-2-$C_{20:4}$-GPC respectively. The values represent means ± S.D. of three experiments conducted in triplicate.

Concn.	Activity (% of control) 1-$C_{16:0}$-2-$C_{18:2}$ PC	1-$C_{16:0}$-2-$C_{20:4}$ PC
0	100	100
1 μM	103 ± 2	41 ± 8
0.1 mM	95 ± 2	58 ± 9
1 mM	68 ± 6	51 ± 5

Table 3. Effect of nucleotides on guinea-pig heart microsomal PLA_1 activity

PLA_1 activity was assayed in the presence of various nucleotides. No nucleotides were added to control tubes. The assays were performed as described in the Materials and methods section. The specific activities of PLA_1 with no addition were 55 ± 7 and 34 ± 5 nmol of LPC formed/h per mg of protein with 1-$C_{16:0}$-2-$C_{18:2}$-GPC and 1-$C_{16:0}$-2-$C_{20:4}$-GPC respectively. The values represent means ± S.D. of three experiments each performed in triplicate.

Addition	Activity (% of control) 1-$C_{16:0}$-2-$C_{18:2}$ PC	1-$C_{16:0}$-2-$C_{20:4}$ PC
None	100	100
1 mM-GTP	99 ± 8	113 ± 18
1 mM-GDP[S]	97 ± 14	134 ± 16
1 mM-ATP[S]	100 ± 1	115 ± 5
1 mM-ATP	95 ± 9	103 ± 6
1 mM-GTP[S]	68 ± 6	51 ± 5

was observed with as little as 1 μM. In contrast, a 30 % inhibition of 1-$C_{16:0}$-2-$C_{18:2}$-GPC hydrolysis was only observed at a GTP[S] concentration of 1 mM. Similar results were obtained with 1-$C_{16:0}$-2-$C_{18:1}$-GPC (results not shown). The inhibitory effect of GTP[S] on PLA_1 activity was not restricted to nucleotides from Boehringer Mannheim; similar results were obtained with GTP[S] obtained from Sigma. This inhibitory effect of GTP[S] on the PLA_1 activity was specific, as it was not reproduced by GTP, guanosine 5'-[β-thio]diphosphate (GDP[S]), adenosine 5'-[γ-thio]triphosphate (ATP[S]) or ATP (Table 3). In order to eliminate the possibility that the inhibitory effect of GTP[S] on

Table 4. Effect of cation chelators on GTP[S]-mediated inhibition of PLA_1 hydrolysis

PLA_1 hydrolysis of 1-$C_{16:0}$-2-$C_{18:2}$ PC was assayed in the presence of EDTA (2 mM) + GTP[S] (1 mM), EGTA (2 mM) + GTP[S] (1 mM) or the individual additions. The specific activity of PLA_1 with no additives was 47 ± 5 nmol of LPC formed/h per mg of protein. The values represent means ± S.D. of three experiments performed in triplicate: * $P < 0.005$ compared with EDTA alone.

Addition	Activity (% of control)
None	100
EGTA	93 ± 4
EDTA	84 ± 5
GTP[S]	62 ± 5
EDTA + GTP[S]	73 ± 7*
EGTA + GTP[S]	68 ± 2

Table 5. Effect of GDP[S] on GTP[S]-mediated inhibition of PLA_1 hydrolysis of 1-$C_{16:0}$-2-$C_{18:2}$ PC

Microsomes were incubated with GDP[S] (1 mM) for 5 min. Subsequently GTP[S] (1 mM) was added, and the reaction was initiated by addition of substrate. The reaction conditions and analysis of products are described in the Materials and methods section. The specific activity of PLA_1 with no addition was 44 ± 4 nmol of LPC formed/h per mg of protein. The results represent means ± S.D. of three experiments each done in triplicate: * $P < 0.005$ compared with no addition.

Addition	Activity (% of control)
None	100
GTP[S]	72 ± 10*
GDP[S]	96 ± 6
GDP[S] + GTP[S]	96 ± 15

Table 6. Effect of heating on PLA_1 activity in guinea-pig heart microsomes

PLA_1 activity was assayed after heating heart microsomes at 60 °C for the indicated times, followed by cooling to 4 °C in an ice bath for 5 min. The enzyme assays were performed as described in the Materials and methods section. The results represent means ± S.D. of two experiments each performed in triplicate.

Heating period (min)	Activity (% of control) 1-$C_{16:0}$-2-$C_{18:2}$ PC	1-$C_{16:0}$-2-$C_{20:4}$ PC
0	100	100
1	7 ± 0.2	83 ± 13
2	10 ± 0.2	84 ± 3
4	15 ± 5	83 ± 12
8	13 ± 8	64 ± 4
12	7 ± 2	20 ± 4

PLA_1 activity is due to cations that may be present in the nucleotide preparation, we examined the effect of GTP[S] on the hydrolysis of 1-$C_{16:0}$-2-$C_{18:2}$-GPC in the presence of 2 mM-EDTA or 2 mM-EGTA. The results (Table 4) show that the inhibitory effect of GTP[S] was still observed in the presence of both cation chelators. EGTA (2 mM) did not affect the activity of the enzyme, but EDTA (2 mM) caused an inhibition of the enzyme. The incubation of EDTA or EGTA with GTP[S] resulted in a decrease in the rate of PC hydrolysis, compared with rates

obtained with the chelators alone. These results suggest that the inhibition observed in the presence of GTP[S] is not due to cations. We also investigated whether the inhibitory effect of GTP[S] on PLA_1 hydrolysis could be prevented by GDP[S]. 1-$C_{16:0}$-2-$C_{18:2}$-GPC was used in these experiments. Microsomes were incubated with 1 mM-GDP[S] for 5 min before addition of 1 mM-GTP[S]. The results of these experiments (Table 5) clearly show that GDP[S] blocked the inhibition of PLA_1 hydrolysis by GTP[S]. Taken together, the above results provide strong evidence implicating the involvement of G-proteins in inhibition of the PC-hydrolysing PLA_1 activity, but definite proof will have to await the purification and reconstitution of the components. It is worth noting that evidence for G-protein-mediated inhibition of PLA_2 activity has been previously reported [17].

The differential effect of GTP[S] on the hydrolysis of 1-$C_{16:0}$-2-$C_{18:2}$- and 1-$C_{16:0}$-2-$C_{20:4}$-GPC was unexpected, and its physiological significance is not known. However, if a single enzyme hydrolyses both substrates, this would imply the existence of two distinct G-proteins, with different affinities for GTP[S], which modulate the activity of the enzyme for different substrates. On the other hand, hydrolysis of each substrate by a distinct enzyme might mean the differential effect of GTP[S] could be due to differences in the affinities of a single G-protein for the two enzymes. The possible hydrolysis of 1-$C_{16:0}$-2-$C_{18:2}$-GPC and 1-$C_{16:0}$-2-$C_{20:4}$-GPC by distinct enzymes was investigated by assessing the stability of the PLA_1 activity to heating at 60 °C (Table 6). After heating for 1 min, about 90% of the 1-$C_{16:0}$-2-$C_{18:2}$-GPC-hydrolysing activity had been lost. Experiments with 1-$C_{16:0}$-2-$C_{18:1}$-GPC yielded similar results (not shown). Coincidentally, the effect of GTP[S] on these two substrates was also similar. In contrast, heating for up to 4 min resulted in the loss of only 20% of the 1-$C_{16:0}$-2-$C_{20:4}$-GPC hydrolysing activity, and after 8 min almost 40% of the activity was still present. Although these results do not conclusively demonstrate the presence of two distinct enzymes, they do provide strong evidence for just such enzymes which presumably differ in their specificity for different molecular species of PC. Thus there appears to be a distinct PLA_1 for the hydrolysis of 1-$C_{16:0}$-2-$C_{18:2}$- and 1-$C_{16:0}$-2-$C_{18:1}$-GPC and another for 1-$C_{16:0}$-2-$C_{20:4}$-GPC. Differential regulation of the PLA_1 activities would lead to the production of different species of 1-lyso-2-acyl-GPC, which would be hydrolysed by the $LPLA_2$ to release different fatty acid species. Two PLA_1 activities in rat heart with different susceptibilities to heat have been reported [13], but all assays were conducted with 1-$C_{16:0}$-2-$C_{18:1}$-GPC, and hence the substrate preferences of the two are unknown. The probable existence of more than one PLA_1 would lead us to postulate that a single G-protein modulates the activities of both enzymes; however, we cannot rule out the involvement of separate G-proteins.

The sequential action of the cholineglycerophospholipid-hydrolysing PLA_1 and $LPLA_2$ characterized in the tissue would result in the selective release of fatty acids; however, this release would be inhibited upon activation of the PLA_1-modulating G-protein by agonists. Thus the physiological role of the enzymes would not be to release fatty acids in response to agonists, but rather to furnish resting cells with fatty acids for the synthesis of cyclo-oxygenase and lipoxygenase metabolites. It is worth noting that 13-OH-$C_{18:2}$, a lipoxygenase metabolite of linoleic acid, is synthesized under resting conditions, but not in stimulated cells [21–23]. Although it has been suggested that triacylglycerols are the source of the released linoleic acid [23], the evidence is not conclusive. It does not rule out release of linoleic acid from phospholipids followed by replenishment of the fatty acid in the phospholipid fraction by a transfer from triacylglycerols.

In conclusion, the present study has presented evidence that strongly suggests that the activities of phospholipases A_1 like those of other phospholipases, A_2 [7,8], C [9,19] and D [10,11], may be regulated by G-proteins.

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