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# PHOSPHOLIPASE A AND LYSOPHOSPHOLIPASE ACTIVITIES IN ISOLATED FAT CELLS: EFFECT OF CYCLIC 3',5'-AMP

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SUMMARY

1. Homogenates of fat cells, isolated from rat epididymal fat pads, were shown to contain phospholipase A and lysophospholipase activities.

2. The highest phospholipase A activity was found at pH 8, but a separate optimum of lower activity was detected at about pH 5.

3. By using 1-[9,10-$^3H_2$]palmityl-2-[1-$^{14}$C]linoleyl-glycero-3-phosphorylethanolamine it could be demonstrated that fatty acid ester linkages at both position 1 and 2 of the phosphatidylethanolamine were hydrolyzed at pH 8.

4. Lipolysis at both positions of the phosphoglyceride was stimulated by cyclic 3',5'-AMP.

## INTRODUCTION

Isolated fat cells from epididymal fat tissue are well known to contain large amounts of triglycerides. In recent years several studies have been undertaken on the catabolism of these triglycerides. As a result of these studies the presence of a hormone-sensitive lipase in isolated fat cells is well-established[1-3]. Hormonal control of this lipolytic activity is mediated inside the cell through cyclic 3',5'-AMP[4,5]. The present study was carried out to investigate whether fat cells contain other lipolytic enzymes that could be influenced by cyclic 3',5'-AMP. Our data clearly indicate the presence of phospholipase A and lysophospholipase activities in homogenates of isolated fat cells. Phospholipase A, but not lysophospholipase, activity was stimulated significantly in the presence of cyclic 3',5'-AMP. This suggests that also hydrolysis of phospholipids in isolated fat cells can be controlled by those hormones which promote the accumulation of cyclic 3',5'-AMP.

## MATERIALS AND METHODS

### *Preparation of isolated fat cells*

Fat cells were isolated by the procedure of RODBELL[3] from epididymal fat pads

of rats fed *ad libitum*. The fat cells were suspended in 0.1 M Tris–maleate buffers of the required pH value and homogenized at 0° in a glass Potter–Elvehjem tube fitted with a teflon pestle. The suspension of broken cells was centrifuged for 2 min at $400 \times g$ to remove the bulk of fat. The infranatant containing mitochondria, microsomes and the soluble protein fraction was used as the enzyme source.

Protein was measured according to the method of Lowry *et al.*[6].

*Substrates*

[$^{32}$P]Phosphatidylethanolamine was prepared biosynthetically from rat liver as described previously[7].

1-Acyl-[$^{32}$P]phosphatidylethanolamine was obtained by degradation of [$^{32}$P]-phosphatidylethanolamine with phospholipase A from crude *Crotalus adamanteus* venom and purified from the reaction mixture by thin-layer chromatography.

1-Acyl-2-[1-$^{14}$C]linoleyl-glycero-3-phosphorylethanolamine was prepared by acylation of 1-acyl-glycero-3-phosphorylethanolamine with [1-$^{14}$C]linoleic acid by the acyltransferases of rat liver microsomes[7]. The purified product contained the [1-$^{14}$C]-linoleate for 88% in the 2-position as could be deduced from degradation experiments with phospholipase A from *C. adamanteus*. 2-Acyl-glycero-3-phosphorylethanolamine was prepared by degradation of phosphatidylethanolamine with purified mold lipase as described by SLOTBOOM *et al.*[8]. The product was purified from the reaction mixture on a Sephadex-LH20 column and acylated with [9,10-$^{3}H_2$]palmitate in the presence of rat liver microsomes to yield 1-[9,10-$^{3}H_2$]palmityl-2-acyl-glycero-3-phosphoryl-ethanolamine. Degradation of this product with phospholipase A showed it to contain the [9,10-$^{3}H_2$]palmitate for 82% at the 1-position.

[1-$^{14}$C]Linoleic acid and [9,10-$^{3}H_2$]palmitate were purchased from the Radiochemical Centre, Amersham.

Cyclic 3′,5′-AMP was obtained from Boehringer Mannheim. Collagenase was a product of Worthington, U.S.A.

*Incubation procedure*

Sonicated radioactive substrates were incubated with aliquots of the infranatant from homogenized fat cells at 37° in a shaking waterbath. Reactions were stopped by the addition of 3 vol. of chloroform–methanol (1:2, v/v). Lipids were extracted according to the method of BLIGH AND DYER[9], followed by two washes with chloroform. The combined chloroform extracts were evaporated to dryness under reduced pressure and the lipid residue was redissolved in a known volume of chloroform–methanol (1:2, v/v). Aliquots of these solutions were then applied to silica gel G thin-layer plates which were developed with chloroform–methanol–water (65:35:4, by vol.), to separate lysophosphatidylethanolamine, phosphatidylethanolamine and free fatty acids. Lipids were made visible by slightly staining with iodine and radioactivity was localized by scanning the plates with a Berthold LB 2721 thin-layer scanner. $^{14}$C radioactivity was quantitatively determined by scraping the spots into scintillation vials containing 16 ml of a dioxane scintillation mixture as described by SNYDER[10]. Lipids containing both $^{3}$H and $^{14}$C were analysed by using a modification of the procedure of ARVIDSON[11]. The radioactive lipids were scraped into glass centrifuge tubes and extracted 3 times with 3 ml of a mixture of chloroform–methanol–acetic acid–water (50:39:1:10, by vol.). To each eluate 3 ml of 4 M $NH_4OH$ was added. After thorough

mixing and centrifugation the upper phase was removed and the chloroform layer was washed once with 3 ml of methanol–water (1:1, v/v). The chloroform phase was transferred into a scintillation vial, evaporated to dryness, and dissolved in a scintillation mixture consisting of 0.4% 2,5-diphenyloxazole and 0.05% 7-bis-(2-phenyloxazolyl)benzene in toluene. Radioactivity measurements were done in a Packard Tricarb scintillation spectrometer Model 3003. This procedure assured recoveries of 90–95% of the $^{3}H$ and $^{14}C$ radioactivity applied to the thin-layer plate.

[$^{32}P$]Glycero-3-phosphorylethanolamine was identified by paper chromatography on Whatman No. 1 paper using propanol–ammonia–water (6:3:1, by vol.) as a solvent system and authentic glycero-3-phosphorylethanolamine as a reference substance. Phosphorus-containing compounds were stained by spraying the paper with 60% $HClO_4$–1 M HCl–4% ammonium molybdate–water (5:10:25:60, by vol.).

## RESULTS AND DISCUSSION

In order to investigate whether phospholipase activity was present in the fat cells, 1-acyl-2-[1-$^{14}C$]linoleyl-glycero-3-phosphorylethanolamine was incubated with homogenized fat cells in buffers of various pH values. When the decrease in the labelled substrate was plotted as a function of the pH of the incubation medium, a curve showing two maxima resulted (Fig. 1). Maximal phospholipase activity was measured

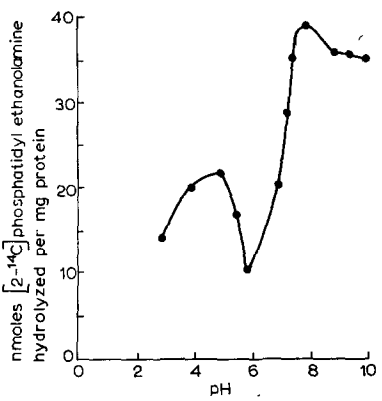


Fig. 1. The effect of pH on the hydroysis of phosphatidylethanolamine by homogenized fat cells. The incubation mixture consisted of 150 $\mu$moles of Tris–maleate of the indicated pH values, 1.5 $\mu$moles of $CaCl_2$, 50 nmoles of 1-acyl-2-[1-$^{14}C$]linoleyl-glycero-3-phosphorylethanolamine and 0.5 mg of fat cells protein in a total volume of 1.5 ml. After a 30-min incubation the lipids were extracted and analyzed as described under MATERIALS AND METHODS.

at alkaline pH (about pH 8), whereas a peak of lower activity was found at acidic pH values (about pH 5). Total hydrolysis of [$^{14}C$]phosphatidylethanolamine at pH 8.0 was linear with protein up to 1 mg. Under these conditions, about 20% of the substrate was being hydrolyzed during the incubation. A similar curve was obtained when homogenates of whole fat pads instead of homogenized fat cells were used, indicating that the collagenase treatment had no effects on the observed enzymic activities.

A separate analysis of the products of the hydrolysis of 1-acyl-2-[1-$^{14}C$]linoleyl-

glycero-3-phosphatidylethanolamine showed that both the free fatty acid and the monoacyl-glycero-3-phosphorylethanolamine were $^{14}C$ labeled. Although this result was obtained both at pH 5 and at pH 8, further experiments were confined to the more active phospholipase activity at pH 8.

The production of [$^{14}C$]lysophosphatidylethanolamine and [$^{14}C$]fatty acid suggested the presence of both phospholipase $A_1$ and $A_2$, but these results could be effected also by the combined action of a phospholipase $A_1$ and a lysophospholipase hydrolyzing the 2-acyl-glycero-3-phosphorylethanolamine. For this reason experiments with [$^{32}P$]phosphatidylethanolamine and 1-acyl-[$^{32}P$]glycero-3-phosphorylethanolamine were carried out. As shown in Table I, about 10% of the initial substrate radioactivity is recovered in the water-soluble glycero-3-phosphorylethanolamine, indicating the presence of a lysophospholipase activity. The addition of sodium deoxycholate prevented the formation of glycero-3-phosphorylethanolamine nearly completely. Inhibition of lysophospholipase activity by deoxycholate has also been observed with this enzyme from other sources[12]. Consequently, in the presence of deoxycholate, the production of [$^{32}P$]lysophosphatidylethanolamine from [$^{32}P$]phosphatidylethanolamine was increased, thus enabling a more direct proof of the presence of phospholipase $A_1$ and $A_2$. In such experiments homogenized fat cells were incubated with 1-[9,10-$^3H_2$]palmityl-2-[1-$^{14}C$]linoleyl-glycero-3-phosphorylethanolamine. Isolation of the lysophosphatidylethanolamine formed during such incubations and comparison of its $^3H/^{14}C$ ratio with the $^3H/^{14}C$ ratio in the original doubly labeled phosphatidylethanolamine should provide information on the ratio of 1-acyl- and 2-acyl-lysophosphatidylethanolamine formed by homogenized fat cells.

TABLE I

INFLUENCE OF SODIUM DEOXYCHOLATE ON THE HYDROLYSIS OF [$^{32}P$]PHOSPHATIDYLETHANOLAMINE AND [$^{32}P$]LYSOPHOSPHATIDYLETHANOLAMINE BY HOMOGENIZED FAT CELLS

Incubation conditions were as described in the legend to Fig. 1, but were carried out at pH 8.0 and contained when indicated 1.0 mg sodium deoxycholate. The results are expressed as percent of the original substrate and are the mean values of three determinations.

| *Substrate* | *Sodium deoxycholate* | *% of $^{32}P$ radioactivity* | | |
|---|---|---|---|---|
| | | *Phosphatidyl-ethanolamine* | *Lysophosphatidyl-ethanolamine* | *Glyceryl-phosphoryl-ethanolamine* |
| Phosphatidylethanolamine | − | 78.7 | 10.9 | 10.4 |
| | + | 74.8 | 25.0 | 0.2 |
| Lysophosphatidylethanolamine | − | − | 65.0 | 12.0 |
| | + | − | 80.0 | 0.1 |

Incubation of a doubly labeled phosphatidylethanolamine with a $^3H/^{14}C$ ratio of 0.88 in the presence of deoxycholate produced a lysophosphatidylethanolamine fraction with an isotopic ratio of 1.15 ± 0.20 indicating that the lysophosphatidylethanolamine fraction consisted of about 60% 1-acyl isomer. Experiments in the absence of deoxycholate yielded a lysophosphatidylethanolamine mixture having an isotopic ratio of 0.52 ± 0.08 and hence consisting of about 30% 1-acyl-lysophosphatidylethanolamine (Table II). This presence of less of the 1-acyl isomer in the absence of deoxycholate theoretically can be explained by a preferential hydrolysis of the 1-acyl-lysophosphatidylethanolamine by the lysophospholipase. In this respect it is of

interest to note that the lysophospholipase of rat liver hydrolyzed 1-acyl lysolecithin at a rate twice that observed for 2-acyl lysolecithin[13]. On the other hand, deoxycholate may have different effects on phospholipase $A_1$ as compared to phospholipase $A_2$, thus producing a lysophosphatidylethanolamine fraction consisting of a larger part of the 1-acyl isomer.

TABLE II

HYDROLYSIS OF 1-[9,10-$^3H_2$]PALMITYL-2-[1-$^{14}$C]LINOLEYL-GLYCERO-3-PHOSPHORYLETHANOLAMINE BY HOMOGENIZED FAT CELLS

The incubation mixture contained 51 nmoles of doubly labeled phosphatidylethanolamine (50 000 counts/min of $^3H$ and 56 000 counts/min of $^{14}C$), 1.5 $\mu$moles of $CaCl_2$, 1.0 mg of sodium deoxycholate (when indicated) and 1.0 mg of fat cell protein in a total volume of 1.5 ml of a 0.1 M Tris–maleate buffer, pH 8.0. After 30-min incubation at 37°, lipids were extracted, purified, and used for the determination of $^3H$ and $^{14}C$ radioactivity as described under METHODS. Control experiments showed that degradation of the doubly labeled phosphatidylethanolamine ($^3H/^{14}C$ ratio = 0.88) with *C. adamanteus* phospholipase $A_2$ yielded 1-acyl-glycero-3-phosphorylethanolamine with a $^3H/^{14}C$ ratio of 6.0 whereas the fatty acids released (and hence the 2-acyl-glycero-3-phosphorylethanolamine) exhibited a $^3H/^{14}C$ ratio of 0.2. The reported values are the average of 6 experiments ± S.E. The statistical analysis of the results was done by paired "*t*" test.

| *Sodium deoxycholate* | *$^3H/^{14}C$ ratio of lysophosphatidylethanolamine formed by fat cells* |
|---|---|
| − | 0.52 ± 0.08 |
| | $P < 0.05$ |
| + | 1.15 ± 0.20 |

To study a possible influence of cyclic 3′,5′-AMP, a series of experiments was designed in which homogenized fat cells were incubated in the presence of 1-[9,10-$^3H_2$]-palmityl-2-[1-$^{14}$C]linoleyl-glycero-3-phosphorylethanolamine with and without the addition of cyclic 3′,5′-AMP. To inhibit lysophospholipase activity and therefore to exclude a possible influence of cyclic 3′,5′-AMP on the lysophospholipase, sodium deoxycholate was added to the incubation mixtures. The activity of phospholipase $A_1$ and $A_2$ was detected by measuring the $^{14}C$ and $^3H$ radioactivity in the lysophosphatidylethanolamine formed during such incubations. As shown in Table III, cyclic 3′,5′-

TABLE III

EFFECT OF CYCLIC-3′,5′ AMP ON THE HYDROLYSIS OF 1-[9,10-$^3H_2$]PALMITYL-2-[1-$^{14}$C]LINOLEYL-GLYCERO-3-PHOSPHORYLETHANOLAMINE BY HOMOGENIZED FAT CELLS

Incubation conditions were similar to those described in the legend of Table II with the addition of 1 mg of sodium deoxycholate. The amount of cyclic 3′,5′-AMP, when present, was 1 mg per incubation flask. Control experiments showed that degradation of the doubly labeled phosphatidylethanolamine ($^3H/^{14}C$ = 0.84) with *C. adamanteus* phospholipase $A_2$ yielded 1-acyl-glycero-3-phosphorylethanolamine with a $^3H/^{14}C$ ratio of 8.3, whereas the fatty acids released exhibited a $^3H/^{14}C$ ratio of 0.3. Each determination was run by triplicate and the values are the mean of 7 experiments ± S.E. The statistical analysis of the results was performed by paired "*t*" test.

| *Cyclic 3′,5′-AMP* | *Percentage of the original substrate hydrolyzed* | |
|---|---|---|
| | *[$^{14}C$]Lysophosphatidylethanolamine* | *[$^3H$]Lysophosphatidylethanolamine* |
| − | 5.5 ± 1.6 | 8.5 ± 2.6 |
| + | 16.3 ± 4.9 | 21.5 ± 5.8 |

AMP stimulates both phospholipase $A_1$ and $A_2$ activities to a similar extent. Phosphatidylethanolamine hydrolysis was also stimulated several fold by cyclic 3′,5′-AMP in the absence of sodium deoxycholate. 5′-AMP did not affect phosphatidyl-

ethanolamine hydrolysis, neither in the presence nor in the absence of sodium deoxycholate. Both cyclic 3′,5′-AMP and 5′-AMP had no effect on the deacylation of 1- 1-$^{14}$C]-palmitoyl lysolecithin by the lysophospholipase in homogenized fat cells.

The stimulation of phospholipases $A_1$ and $A_2$ by cyclic 3′,5′-AMP suggests that the activity of these enzymes may be controlled by those hormones that promote cyclic 3′,5′-AMP formation in fat cells. Since lipases have been shown to act on the fatty acid ester linkage at the 1-position of phosphoglycerides[14], it cannot be excluded at present that the hydrolysis of fatty acid from the 1-position of phosphatidylethanolamine was actually catalysed by the hormone-sensitive lipase of fat cells. Particularly interesting is the fact that also hydrolysis at the 2-position of phosphatidylethanolamine is stimulated by cyclic 3′,5′-AMP. Phospholipids contain higher amounts of polyunsaturated fatty acids than neutral lipids, especially at the 2-position of the glycerol moiety. Stimulation of a phospholipase $A_2$ by cyclic 3′,5′-AMP may therefore yield an increased amount of these polyunsaturated fatty acids during hormone-stimulated lipolysis. CHRIST AND NUGTEREN[15] have shown that triglycerides from rat epididymal adipose tissue contain 1.7% of arachidonic acid. Yet, the free fatty acids released during basal and hormone-stimulated lipolysis existed for 8.7 and 4.7% of arachidonic acid. This would imply either a preferential hydrolysis of this polyunsaturated fatty acid from triglycerides or a release of arachidonic acid during lipolysis from a precursor containing higher quantities of arachidonate, possibly that 2-position of phosphoglycerides.

Several authors[15,16] have shown that prostaglandin E can be formed in epididymal fat pads from the polyunsaturated fatty acid precursors that are released during lipolysis. Biologically active prostaglandins E were found to inhibit both the accumulation of cyclic 3′,5′-AMP and the release of free fatty acids, thereby suggesting a physiological role of prostaglandins E in the regulation of lipolysis. However, the possible role of a hormone-sensitive phospholipase $A_2$ in this chain of events has not been appreciated before.

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