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High-performance liquid chromatographic study of the regulation of phospholipid metabolism in cultured adrenocortical cells

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Abstract

A rapid high-performance liquid chromatographic (HPLC) method for the separation of phospholipids was developed for minute samples of total lipids (*ca.* 200 μg). The method was applied to the study of the phospholipid metabolism in adrenocortical cell cultures. A complete separation of the different cellular phospholipid classes was achieved in 40 min. Good resolution of the phospholipid peaks was obtained, which allowed the collection of each individual class of phospholipids for further analysis of radioactivity and fatty acid composition by gas chromatography. When cells were incubated with [U-^{14}C]glycerol or [U-^{14}C]palmitate the bulk of the radioactivity was found in cellular phosphatidylcholines. Exogenous phospholipids were incorporated into cellular lipids to a large extent, however without an increase in the cellular phospholipid content. 12-O-Tetradecanoyl-phorbol-13-acetate induced a 20% increase in the polyunsaturated fatty acid content of the cellular phosphatidylethanolamines, but no change was detected in the cellular phosphatidylcholines. The developed method is well-suited to the study of the phospholipid metabolism in adrenocortical cells where the phospholipid metabolism is closely linked to the specialized functions of the cells.

1. Introduction

Several important cell functions depend on phospholipids which are not only structural cell membrane constituents, but also form a source of biologically active molecules after phospholipase hydrolysis. Especially diacylglycerol (DAG), phosphatidic acid (PA) and inositol-triphosphate act as second messengers [1–5]. Phospholipids (PLs) can also act directly on membrane bound enzymes [6,7], especially on those involved in the steroidogenic pathway [8,9]. Cellular PLs are subject to metabolic modifications induced by exogenous signals. Therefore a precise analysis of each class of cellular PLs and their fatty acid composition is needed to determine the changes in their metabolism that cannot be shown by a total-PL analysis. Thin-layer chromatography (TLC) can be used for PL separation [10,11], and good separation of all PLs is obtained by two-dimensional TLC. However identification and quantification are difficult to perform. Loss of polyunsaturated fatty acids as a result of autooxidation represents another drawback of TLC

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[12]. Kaduce *et al.* [13] extracted PLs from bovine aortic endothelial cells and separated the polar PLs from each other by HPLC. However diphosphatidylglycerol (DPG) could not be separated from the neutral lipids (NLs). Juaneda and Rocquelin [14] achieved separation between different PL classes, but this method needed a preceding separation of the PLs from the non-phosphorus lipids to avoid their overlap with the DPG fraction and was appropriate only for large samples (3 to 7 mg of total PL).

This work describes an HPLC method, based upon modifications of the method of Juaneda and Rocquelin [14], which allows direct and complete separation of all PL classes, including DPG and NL, from minute samples (150–250 μg of total lipids) extracted from cultured cells. The method is shown to be especially suitable for the study of PL metabolism and turnover in cultured adrenocortical cells where DPG, PC and PE play an important role in the regulation of steroidogenesis.

2. Experimental

2.1. Reagents

HPLC-grade 2-H-propanol and hexane were obtained from BDH Merck (Darmstadt, Germany). Diphosphatidylglycerols (DPG), phosphatidyl-ethanolamines (PE), phosphatidylinositols (PI), phosphatidylserines (PS), phosphatidylcholines (PC) and 12-O-tetradecanoylphorbol-13-acetate (TPA) were supplied by Sigma (St. Louis, MO, USA). [U-^{14}C]Glycerol, [U-^{14}C]palmitic acid and [methyl-^{14}C]choline-PC were purchased from New England Nuclear (Boston, MA, USA). Ham's F10 medium and phosphate buffered saline (PBS) were from Gibco BRL (Cergy-Pontoise, France).

2.2. Cell culture and lipid extraction

Y1 mouse adrenocortical tumor cells obtained from ICN-Flow (Irvine, UK) were grown to confluency in 25-cm^2 dishes in Ham's F10 medium supplemented with 5% foetal bovine serum, 5% newborn calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin. Monolayers of Y1 cell cultures were incubated in serum and protein free medium [15]. This medium was supplemented with [U-^{14}C]glycerol or dextran [U-^{14}C]palmitate complex [15] to study their incorporation into cellular lipids. In order to study the incorporation of exogenous PL into the cells, liposomes prepared from soya PC and [methyl-^{14}C]choline-PC or from bovine heart DPG, were added to the basal medium. The effect of TPA on cellular PL metabolism was studied by adding this compound (100 n*M*) to the basal culture medium. At different times, cells were washed three times with PBS and scraped off with a rubber spatula in 1 ml of 0.1 *M* HCl solution. The remaining cells were removed by washing the dishes twice with 1 ml of methanol. Total lipids were extracted with 4 ml of chloroform according to the method of Folch *et al.* [16]. The chloroform phase containing the total lipids was recovered for chromatographic analysis.

2.3. High-performance liquid chromatography

A Kontron (Zurich, Switzerland) D450 liquid chromatographic system was employed, consisting of a Model 420 pump, and a Model MT2 system controller. PLs were detected using a Model 430 variable wavelength detector set at 206 nm. PL separation was achieved with a Merck (Darmstadt, Germany) 250 × 4 mm I.D. column packed with 5 μm LiChrosorb Si 60 particles. The same column was also used for collection of PL peaks. For routinè PL separations, 150 to 250 μg of total cellular lipids were taken up in 60 μl of 2-propanol–hexane (8:6, v/v). A 40-μl aliquot of this solution was injected onto the HPLC column. PLs were eluted at room temperature with a gradient of 2-propanol–hexane–water from 55:42:3 to 52:39:9 (v/v/v) at a flow-rate of 0.8 ml/min during 15 min followed by an isocratic run with the 52:39:9 mixture for 25 min. The solvent gradient was obtained using two pumps, one delivering a 2-propanol–hexane mixture (57:43, v/v) and the other water.

2.4. Lipid analysis and quantification

For several measurements each PL fraction was collected, dried under nitrogen at 60°C and kept in chloroform–methanol (1:1, v/v) until analysis. The recovered PLs were aliquotted for measurement of radioactivity, quantitative phosphorous analysis, and analysis of the fatty acid content. The recovery of radioactivity after HPLC separation, calculated by the sum of the amounts found in the individual fractions, was *ca.* 90%. Labelled lipid fractions were counted in a Beckman (Palo Alto, CA, USA) Model LS6000IC counter using 4 ml of a mixture of Permafluor–toluene (1:9, v/v). Quantitative phosphorus analysis was carried out using the method of Duck-Chong [17]. For the fatty acid analysis of the PLs, 30 μg of the dry and purified lipid fraction were transesterified with 1 ml of methanol and 50 μl of pure sulfuric acid and kept under nitrogen at 100°C for 2 h. After cooling, 1 ml of K_2CO_3 (5%) was added and the methyl esters were removed by extraction with hexane (2 ml). Quantitative analysis by gas chromatography (GC) was performed with a Packard-Becker (Delft, Netherlands) Model 427 chromatograph equipped with an SE30 25 m × 0.3 mm I.D. (Spiral, Couternon/Dijon, France) capillary column, using heptadecanoic acid methyl ester as internal standard. Polyunsaturated fatty acids were identified as their methyl ester derivatives by comparison with data already published [18] and by gas chromatography–mass spectrometry using a Ribermag-Nermag (Rueil-Malmaison, France) R10-10-C quadrupole instrument. The gas chromatographic conditions were the same as above. The ionization was performed in the electron impact mode at 70 eV.

3. Results

Fig. 1 shows a chromatogram of standard phospholipids. They are completely separated from each other and from the first peak which corresponds to neutral lipids (NLs) originating from sample impurities. The important HPLC parameters were the slope of the solvent gradient, the flow-rate, and the presence of a small amount of water in the mobile phase. Attempts to change the chromatographic parameters given in the Experimental section in order to decrease the time of analysis resulted in a poorer peak separation: overlap of NL and DPG, and of PE and PI. The small amount of water used in this experiment did not impair the easy recovery of the PL peaks by evaporation of the chromatographic fraction under a stream of nitrogen. In fact the small amount of water was needed to elute PI, PS, and PC from the column.

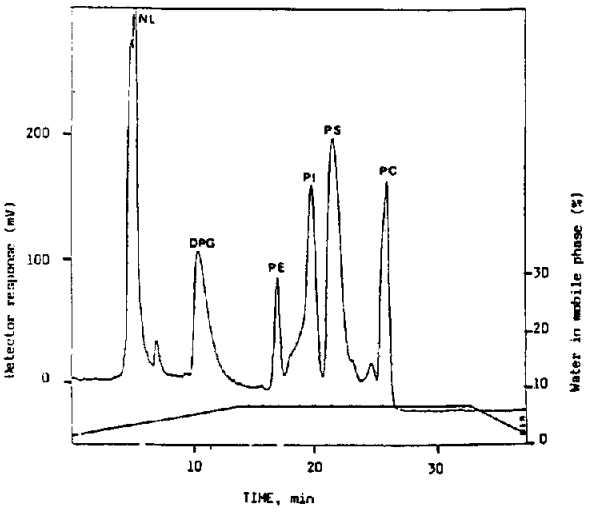

Fig. 1. Separation of a phospholipid standard mixture. Mobile phase: 2-propanol–hexane–water (55:42:2 to 52:39:9, v/v/v in 15 min); flow-rate: 0.8 ml/min; 250 × 4 mm I.D. column packed with 5 μm LiChrosorb Si60 particles; UV detection at 206 nm. NL: neutral lipids; DPG: diphosphatidylglycerol; PE: phosphatidylethanolamine; PI: phosphatidylinositol; PS: phosphatidylserine; PC: phosphatidylcholine. The solvent gradient is shown at the bottom of the chromatogram.

3.1. Cellular lipid separation and detection

Total lipids extracted from $3 \cdot 10^6$ adrenocortical cells were directly injected into the HPLC column. Fig. 2 shows the separation of the various PL classes and the NLs. This last group was analysed separately by TLC and contained triacylglycerols (TAG), diacylglycerols (DAG), free cholesterol (FC), cholesteryl ester

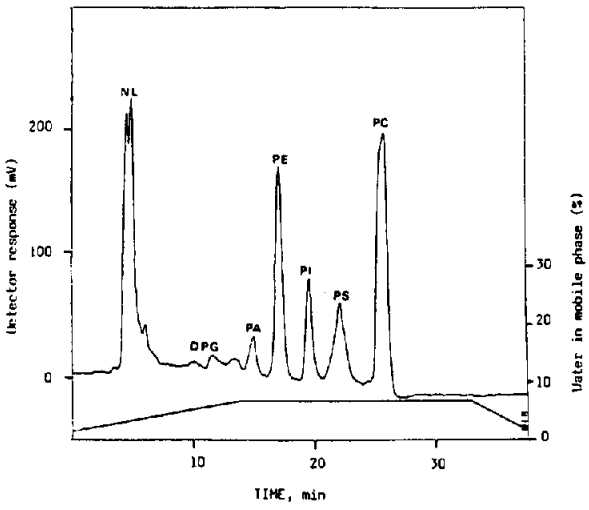

Fig. 2. Separation of adrenocortical cell phospholipids. The lipids to be chromatographed were extracted from a culture containing $3 \cdot 10^6$ cells. The conditions and compound abbreviations were indicated in Fig. 1, except for PA: phosphatidic acid. Each peak was collected and submitted to phosphorus quantitative analysis and individual fatty acid determination by GC.

(CE) and free fatty acids (FFA) (results not shown). Peaks corresponding to DPG, PE, PI, PS and PC were detected at 206 nm. Phosphatidic acid (PA) was eluted between the DPG and PE peaks.

UV detection at 206 nm did not allow the measurement of the molar amount of PL but rather gave a response depending on the quantity of double bonds present in the compound. Measurement of the molar quantities of the main PL classes was achieved by collecting the PL fractions and performing a quantitative phosphorus analysis.

3.2. [U-^{14}C]Glycerol and [U-^{14}C]palmitate incorporation into cellular lipids

Cells incubated with [U-^{14}C]glycerol or [U-^{14}C]palmitate were harvested after 24 h of culture. Cellular radioactivity was mainly incorporated into the lipid fraction (95%). As shown in Table 1, the radioactive precursors were incorporated mainly into the PC fraction, *i.e.* 60 and 72% of the incorporated radioactivity, when cells were incubated with [U-^{14}C]glycerol or [U-^{14}C]palmitate, respectively.

3.3. Exogenous PC or DPG incorporation in adrenocortical cells

Liposomes prepared from soya PC and [methyl-^{14}C]choline-PC were used to study the uptake and metabolism of exogenous PL in

Table 1
Radioactivity incorporated from [U-^{14}C]glycerol or [U-^{14}C]palmitate into cellular lipid classes

Lipid fraction	+[U-^{14}C]glycerol		+[U-^{14}C]palmitate	
	dpm	%	dpm	%
NL	1884 ± 187	3.8	10 737 ± 958	7.4
DPG + PA	1984 ± 267	2.6	6595 ± 195	4.5
PE	6546 ± 589	13.2	12 858 ± 973	8.8
PI	735 ± 145	1.5	1450 ± 857	1.0
PS	4716 ± 377	9.5	8582 ± 934	5.9
PC	34 431 ± 1270	69.4	104 959 ± 1150	72.4
Recovered radioactivity	49 606		144 981	
Injected radioactivity	55 100		166 640	
Recovery	90%		87%	

Y1 adrenocortical cells were incubated with [U-^{14}C]glycerol or [U-^{14}C]palmitate. After 24 h, cellular lipids were extracted and separated by HPLC as described in Experimental. Radioactivity in each fraction was measured by liquid scintillation. Results are given as dpm/mg of protein per 24 h, and are the mean of duplicate experiments.

adrenocortical cells. Control cells without exogenous PL and treated cells were harvested at different times between 3 and 24 h. After separation of the cellular PLs by HPLC, the radioactivity and the phosphorus content of the PC fractions were measured. The kinetics of the uptake of [methyl-^{14}C]choline-PC containing liposomes by cultured adrenocortical cells is shown in Fig. 3A. After a rapid increase in the radioactivity the rate of take-up decreased gradually after 12 h of incubation. However, as shown in Fig. 3B the total amount of PC in liposome-incubated cells was not different from the amount of PC in control cells during the 24-h incubation periods. Consequently, exogenous PC was not accumulated in adrenocortical cells, but an active exchange between exogenous and cellular PC occurred. This exchange seems to be a regulation mechanism which leads to homeostasis of the PL content in the cells. In another experiment the uptake and the metabolic fate of bovine heart DPG introduced into adrenocortical cell cultures as liposomes was studied as a function of incubation time. Table 2 shows the fatty acid composition of DPG, purified by HPLC, from incubated cells and control cells. The most important change was found in the linoleic acid (C18:2) content. The C18:2 content of DPG in incubated cells was increased 2.5-fold compared to control cells after 6 h of incubation. Even after 24 h the C18:2 content of incubated cells remained much higher than that of control cells. This enrichment in C18:2 of cellular DPG may be the basis for the modulation of the activity of the steroidogenic enzymes linked to PL in the bilayer of mitochondrial membranes (results not shown).

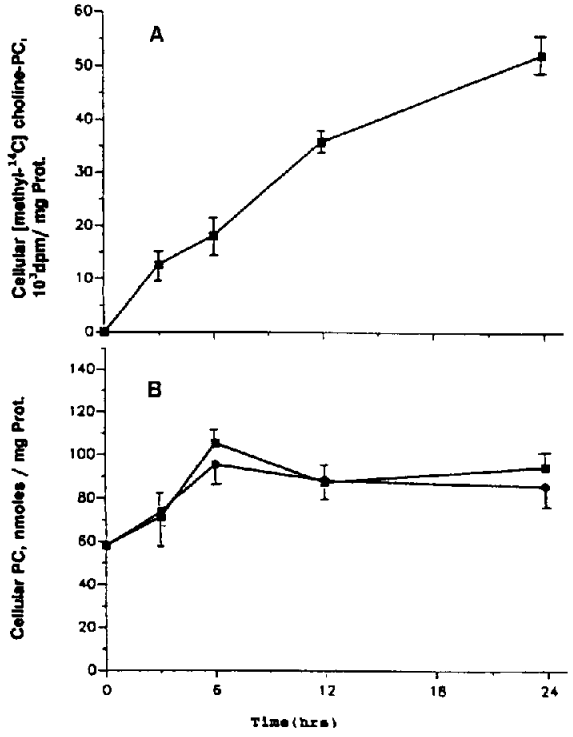

Fig. 3. Transfer of labeled and total phosphatidylcholines from liposomes to Y1 adrenocoartical cells in function of time. Cells were incubated with basal medium alone or supplemented with liposomes prepared with PC from soya bean (33 μM) and [methyl-^{14}C]choline-PC. At indicated times both control cells or incubated cells were harvested, cellular lipids were extracted and chromatographed as described in Fig. 1. In each sample the PC fraction was collected. (A) In incubated cells the radioactivity of the PC fraction was measured, results are given as dpm per mg of cellular protein. (B) PC extracted from control cells (■) or cells incubated with liposomes (●) are given in molar amounts using phosphorus quantitative analysis (nmoles of PC per mg of cellular protein). Values are the mean of duplicate experiments.

3.4. *Effect of phorbol esters (TPAs)*

TPAs have been widely used to induce steroidogenesis in adrenocortical cells [19–22]. Table 3 shows the fatty acid composition of PE and PC. An increase in the degree of unsaturation of PE fatty acid was found in TPA incubated cells (+20%), even though the total PE content remained the same as that found in the control cells. In contrast, no significant changes were detected in the PC fatty acid composition. Changes in the fatty acid composition of PE may also play a role in kinase C activation.

4. Discussion and conclusions

PL metabolism plays an important role in adrenocortical cells. PLs modulate several functions in these cells and especially those linked to steroidogenic enzymes. In the present paper, as

Table 2
Fatty acids of DPG fraction before and after incubation with bovine heart DPG brought as liposomes into adrenocortical cells in culture

Fatty acid	Contents (%, w/w)					
	6 h		12 h		24 h	
	C	DPG	C	DPG	C	DPG
C16:0	11.0	13.9	21.2	13.4	27.4	27.3
C16:1	tr	tr	tr	tr	tr	tr
C18:0	3.3	3.3	6.8	5.2	10.4	7.8
C18:1	23.8	22.3	38.5	32.3	42.0	45.4
C18:2	7.1	14.5	9.3	19.5	13.8	19.4
C22:3	54.8	46.0	24.0	29.5	6.3	tr
Total fatty acids (μg/mg of protein)	15.9	17.5	11.5	11.9	7.9	8.6

Y1 adrenocortical cells were incubated in basal medium alone or in basal medium supplemented with liposomes prepared from bovine heart DPG (33 μM). At indicated times, both control cells or incubated cells were harvested; cellular lipids were extracted and separated as described. The cellular DPG fraction was collected and fatty acid analysis was performed by GC. Fatty acids are expressed as % by weight of total fatty acid content of DPG fraction. Total fatty acids are given as μg/mg of cellular protein. Values are the mean of duplicate experiments, the deviation was less than 5% of the value.

Table 3
Fatty acid composition [in μg/mg of protein per 24 h and in percent (in parentheses)] of PE and PC from adrenocortical cells incubated with phorbol esters

Fatty acid	Composition			
	PE		PC	
	Control	+TPA	Control	+TPA
C16:0	5.4 (18)	tr	17.4 (37)	16.2 (37)
C16:1	0.7 (2)	tr	0.9 (2)	0.7 (2)
C18:0	6.7 (22)	4.8 (15)	6.9 (16)	6.7 (16)
C18:1	1.3 (4)	1.2 (4)	3.7 (8)	3.0 (7)
C18:2	4.7 (16)	4.0 (12)	5.2 (11)	6.7 (16)
X1	–	0.6 (2)	–	–
X2	–	0.6 (2)	–	–
C22:2	2.7 (9)	5.5 (17)	2.9 (9)	2.8 (7)
C22:3	1.6 (5)	4.2 (13)	1.6 (4)	1.7 (4)
C22:4	2.2 (7)	4.3 (13)	1.0 (2)	0.7 (2)
C22:5	0.9 (3)	1.9 (6)	tr	tr
Total	30.5	32.3	45.7	43.3

Y1 adrenocortical cells were incubated in serum and protein free medium alone or serum and protein free medium supplemented with TPA (100 nM) for 24 h. Cellular lipids were extracted and separated by HPLC as described in Experimental. PE and PC fractions were submitted to fatty acids composition analysis by GC. X1, X2: unidentified fatty acids; tr: traces (less than 0.1 μg/mg of protein per 24 h). Values are the mean of duplicate experiments and the deviation was less than 5% of the value.

well as in other works [7], it has been shown that homeostasis of PLs was maintained in adrenal cell cultures even under stimulation by corticotropin or cholera-toxin. Nevertheless some modifications have been observed in the fatty acid moiety of some PL classes [7], showing the importance to perform a detailed analysis of the fatty acid content and composition in each PL class. In this work we have adapted an HPLC method to analyse directly microsamples of lipid extracts from cultured cells. This method, using no preceding purification of the PLs, allowed the complete separation of the different PL classes from each other and from the NLs in a short time (40 min). The good resolution of the PL chromatographic peaks was especially suited for radioactive or non-radioactive peak collection needed in the metabolic studies. Nevertheless the quantitative detection of PL peaks remains difficult. Light-scattering detection is at present widely used in lipid laboratories [23,24] and it allows direct quantitative measurement. In our case UV detection was preferred because it is a less expensive, sensitive and non destructive method: it allowed the recovery of fractions used for measuring the radioactivity and analysis of the fatty acid composition of each PL class before and after incubation. When adrenocortical cells where incubated with [U-^{14}C]glycerol or [U-^{14}C]palmitate, more than 70% of the total radioactivity incorporated into the cells was found in the PC fraction after 24 h. These results indicate that cellular PCs were actively metabolized in adrenocortical cells. Although PC biosynthetic pathways are well known, the process of their catabolism and resynthesis in the cells remains largely unknown [25]. Using this HPLC procedure, we have demonstrated that exogenous DPG and PC were incorporated into cellular lipids to a large extent. The mechanism of incorporation of PC from incubations with liposomes involved an exchange with cellular PL, since no enhancement of the endogenous PL content was observed, although there was an increase in the radioactivity of the intracellular PC. The alteration of the cellular PL fatty acid composition by exogenous PL induced a modulation of steroidogenesis in Y1 adrenocortical cells [26]. Finally, with this HPLC method we have demonstrated that an exogenous effector such as TPA, which is known to alter steroid biosynthesis, can modify the fatty acid composition of cellular PE. Especially in steroidogenic cells, adrenocorticotropic hormone (ACTH) induces changes in the fatty acid composition of some cellular PLs [7]. However the question remains: are the changes in the fatty acid composition following cell stimulation involved in steroid biosynthesis or are the changes in PL and the activation of steroidogenesis independent of the hormonal signals? Several works demonstrated an effect of PL on purified steroidogenic enzymes *in vitro*. In this work we demonstrated that alteration of the fatty acid composition of adrenocortical cell PLs can be induced in intact cells by incubating cultured cells with exogenous PL or effector.

This study was facilitated by using the proposed HPLC method for cellular PL separation. We plan to use this methodology also in further studies on the mode of action of PLs on steroidogenesis in adrenocortical cells in culture.

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