The phospholipid analogue hexadecylphosphocholine inhibits phosphatidylcholine biosynthesis in Madin–Darby canine kidney cells

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The influence of the phospholipid analogue hexadecylphosphocholine on phosphatidylcholine biosynthesis was investigated in Madin–Darby canine kidney (MDCK) cells. It inhibits the incorporation rate of [*methyl*-³H]choline into phosphatidylcholine at a concentration of 50 µM by about 50%. The radiolabelled precursor accumulates in the phosphocholine pool indicating that hexadecylphosphocholine inhibits the formation of phosphatidylcholine via the CDP-choline pathway at the level of the rate-limiting enzyme, CTP:phosphocholine cytidylyl transferase (EC 2.7.7.15). This was verified by the determination of the activity of the enzyme in vitro. In consequence of its inhibitory effect it could be shown that the treatment of MDGK cells for 24 h with 50 µM hexadecylphosphocholine induces alterations of the phospholipid composition. Whereas in treated cells the relative phosphatidylcholine content was decreased from the control level of 36.0±0.9% to 29.9±0.2%; in contrast, the relative content of phosphatidylethanolamine was increased from 19.3±0.9% to 24.3±0.9%.

Phosphatidylcholine biosynthesis; CTP:phosphocholine cytidylyltransferase; Hexadecylphosphocholine; Madin–Darby canine kidney cell

1. INTRODUCTION

In mammalian tissues the most abundant phospholipid is phosphatidylcholine. Its biosynthesis was the subject of several investigations (for review see [1]). Studies on the influence of alkyllysophosphoglycerides on phospholipid metabolism demonstrated that these analogues disturb especially the metabolism of phosphatidylcholine [2]. In contrast to the previously investigated analogues, hexadecylphosphocholine belongs to a group of new antineoplastic substances, the alkylphosphocholines, and is structurally different from the alkyllysophosphoglycerides and acyllysophosphoglycerides (Fig. 1). Hexadecylphosphocholine is only slowly metabolized in vivo [3] and recently it could be shown that this phospholipid analogue inhibits protein kinase C [4,5].

In the present study it was demonstrated that hexadecylphosphocholine interferes with the biosynthesis of phosphatidylcholine. Our results show that it inhibits the formation of phosphatidylcholine via the CDP-choline pathway at the level of the rate-limiting enzyme, CTP:phosphocholine cytidylyltransferase (EC 2.7.7.15). Moreover, the lipid composition of MDCK cells was altered by treatment with 50 µM hexadecylphosphocholine for 24 h.

Abbreviations: BCA, bicinchonicic acid; HPTLC, high-performance thin-layer chromatography; MDCK, Madin-Darby canine kidney; HcPC, hexadecylphosphocholine

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2. MATERIALS AND METHODS

2.1. *Materials*

[*methyl*-³H]Choline chloride (2.8–3.1 TBq/mmol) and [*Methyl*-¹⁴C]phosphocholine (2.04 GBq/mmol) were from Amersham (Braunschweig, Germany). Hexadecylphosphocholine was a gift from Asta Pharma (Frankfurt am Main, Germany). Silica gel 60 HPTLC plates and all solvents and reagents (reagent grade) were purchased from Merck (Darmstadt, Germany). The BCA-kit for protein determination was obtained from Pierce (Weiskirchen, Germany). For quantification of radioactivity a Berthold LB 2821 HR thin-layer chromatography scanner (Berthold; Wildbad, Germany) was used. Dithiothreitol, phenylmethylsulfonylfluoride, miscellaneous lipids and phosphatidylcholine precursors were from Sigma (München, Germany).

2.2. *Cell culture*

Madin-Darby canine kidney (MDCK) cells were grown in Dulbecco's minimal essential medium supplemented with 10% heat-inactivated fetal calf serum, 0.56 g/l glutamine, 110 000 U/l penicillin and 0.1 g/l streptomycin in plastic culture dishes (Nunc, Denmark). Media and culture reagents were obtained from Gibco (Karlsruhe, Germany), the penicillin and streptomycin were from Boehringer (Mannheim, Germany). Cytotoxicity of hexadecylphosphocholine was determined by the method of Culvenor et al. [6].

2.3. *Radiolabelling and extraction of lipids and water-soluble precursors*

[*methyl*-³H]Choline labelling [7.4 × 10⁴ Bq/ml] was initiated after 3 h preincubation of the cells with 50 µM hexadecylphosphocholine or, alternatively, no supplements as control. After the incubation, cells were washed with ice-cold phosphate-buffered saline (pH 7.2), then harvested with a cell lifter (Costar; Cambridge, USA) followed by modified lipid extraction according to Bligh ad Dyer [7]. Shortly after lyophilisation of the cells, 50 µl methanol, 25 µl chloroform and 20 µl water were added. Samples were stirred for 2 min on a vortex mixer and centrifuged at 13 000 × g for 10 min. Phase separation was accomplished by the addition of 25 µl chloroform and 25 µl water. The suspension and centrifugation steps were repeated, and 20 µl of the chloroform phases were applied to silica gel 60 HPTLC plates us-
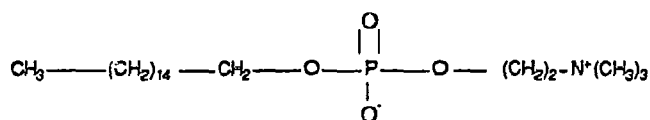
$$CH_3 \text{------} (CH_2)_{14} \text{------} CH_2 \text{------} O \text{------} \overset{\overset{O}{\|}}{\underset{\underset{O^-}{|}}{P}} \text{------} O \text{------} (CH_2)_2 \text{-} N^+(CH_3)_3$$

Fig. 1. Chemical structure of hexadecylphosphocholine

ing a HPTLC applicator (Linomat III; Camag, Berlin, Germany). Seventy μl of the upper phases were lyophilized, dissolved in 20 μl methanol/water (50 : 45, by vol.) and were likewise applied to HPTLC plates.

2.4. Separation and quantification of lipids and water-soluble precursors

Lipids were separated according to Skipsi et al. [8], using the solvent chloroform/methanol/acetic acid/water (25:15:4:2, by vol.). Phosphatidylcholine precursors were separated in methanol/0.6% NaCl/25% aqueous NH$_3$ (8:5:1, by vol.). Radioactivity was quantified by radioscanning. Phospholipids and their precursors were identified by calibrating the scanner with known standards.

2.5. Protein determination

Cellular protein was determined in each sample of the radiolabelling experiments by the BCA-assay [9] using bovine serum albumin as a standard.

2.6. Preparation of MDCK cell homogenate

MDCK cells were harvested as described above in a buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 2 mM dithiothreitol, 0.03% Triton X-100 and 4 mM NaN$_3$. After homogenization by use of a dounce with a loose-fitting pestle (Braun, Melsungen, Germany) the protease inhibitor, phenylmethylsulfonylfluoride, was added to achieve a 1.0 mM final concentration.

2.7. CTP:phosphocholine cytidylyltransferase assay

The cytidylyltransferase activity was measured by a modified method of Sohal and Cornell [10]. The reaction mixture contained 50 mM Tris-HCl, pH 7.4, 0.03% Triton X-100, 100 mM NaCl, 10 mM MgCl$_2$, 3 mM CTP, 1.5 mM [methyl-^{14}C]phosphocholine (specific radioactivity 20 Bq/nmol), liposomes (100 μM phosphatidylcholine/ 100 μM oleic acid) and 5 μl of MDCK cell homogenate in a final volume of 50 μl. After incubation for 30 min at 37°C the reaction was stopped by freezing the samples in liquid nitrogen. The samples were lyophilized, dissolved in 20 μl methanol/water (1:1, v/v) and applied to HPTLC plates. After developing in a solvent system containing methanol/0.6% NaCl/25% aqueous NH$_3$ (8:5:1, by vol.), the radioactivity was determined by radioscanning. One Unit of enzyme activity is defined as nmol of CDP-choline formed per min.

3. RESULTS

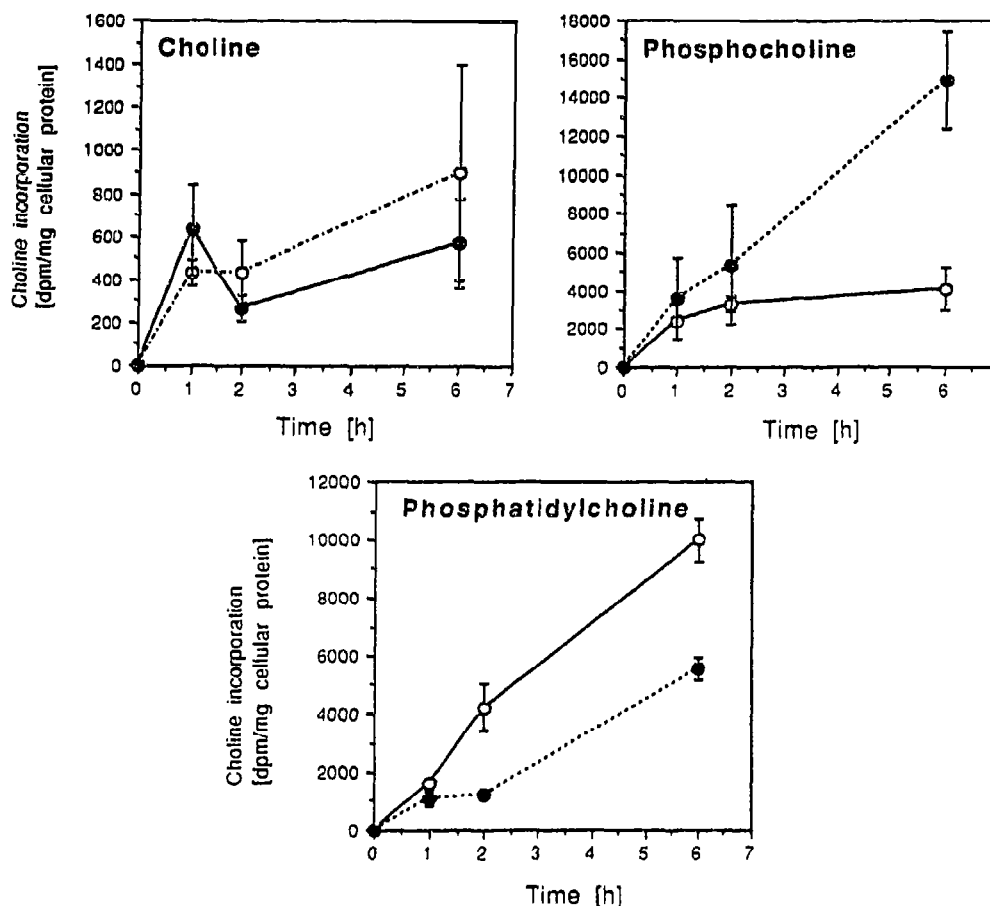It could be shown that hexadecylphosphocholine did

Fig. 2. Effect of hexadecylphosphocholine on the time-dependent incorporation of radiolabelled choline into phosphatidylcholine and its precursors in MDCK-cells. MDCK-cells grown to confluence were preincubated with 50 μM hexadecylphosphocholine for 3 h. Then pulse medium was added containing 7.4 × 10^4 Bq/ml [methyl-^{3}H]choline and 50 μM hexadecylphosphocholine (●). For control experiments no hexadecylphosphocholine was added (O). After different incubation times as indicated in the figure, the cells were mechanically harvested and phosphatidylcholine and phosphatidylcholine precursors were analysed as described in section 2. The values of incorporated radioactivity are given in dpm/mg cellular protein ($n = 3$).
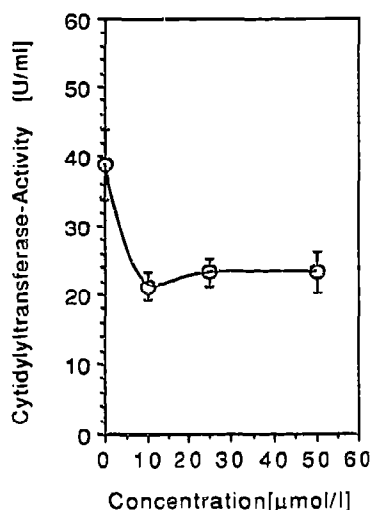
Fig. 3. Effect of hexadecylphosphocholine on cytidylyltransferase (CT) activity in MDCK-cell homogenate. 5 μl of MDCK cell homogenate were used in the assay and various concentrations of hexadecylphosphocholine dissolved in the assay buffer were added to the reaction mixture. The activity is given in U/ml homogenate ($n = 3$).

not show any apparent toxic effect on MDCK-cells up to a concentration of 200 μM. Morphological changes occurred only at concentrations higher than 1 mM hexadecylphosphocholine (data not shown).

To examine the effect of hexadecylphosphocholine on phosphatidylcholine biosynthesis, we measured its influence on [methyl-³H]choline incorporation into phosphatidylcholine precursors and phosphatidylcholine. For this purpose, MDCK-cells were preincubated for 3 h with 50 μM hexadecylphosphocholine, followed by the addition of radiolabelled choline. At different times, as indicated in Fig. 2, the cells were washed and harvested. After an incubation period of 6 h in hexadecyl-phosphocholine-treated cells the incorporation rate of radiolabelled choline into phosphatidylcholine was reduced to about 50% of the control experiments, whereas the incorporation into the phosphocholine pool was increased 3-fold. The incorporation into the

choline pool seemed to be unaffected (Fig. 2) and the incorporation into CDP-choline was not sufficiently detectable in these experiments, because the CDP-choline pool represented less than 1% of the total aqueous metabolites.

The activity of CTP : phosphocholine cytidylyltransferase in MDCK cell homogenate was measured in an assay containing liposomes (Fig. 3). The liposome-stimulated activity was decreased by 45% in the presence of 10 μM hexadecylphosphocholine. It is interesting to note that the inhibitory effect did not increase when higher concentrations of hexadecylphosphocholine were added to the assay buffer. This observation might be due to the critical micellar concentration of hexadecylphosphocholine (CMC), which is in the range of 100 μM (data not shown).

In order to investigate the effect of long-term hexadecylphosphocholine treatment on the lipid composition of MDCK cells we added hexadecylphosphocholine (50 μM) to the culture medium for 24 h and determined the amount of the different lipids in the total lipid extract. The results, shown in Table I, indicate that hexadecylphosphocholine compared with the control experiments, reduces the content of phosphatidylcholine from $36.0 \pm 0.9\%$ to $29.9 \pm 0.2\%$ of total lipid, whereas the content of phosphatidylethanolamine increases from $19.3 \pm 0.9\%$ to $24.3 \pm 0.9\%$. Triacylglycerol, sphingomyelin, phosphatidylserine, phosphatidylinositol and cardiolipin were unaffected. The amount of total lipids was not altered (99.5 ± 9.5 μg/10^6 cells for control cells and 92.9 ± 5.0 μg/10^6 cells for treated cells).

4. DISCUSSION

Phosphatidylcholine biosynthesis occurs mainly via the CDP-choline pathway (Kennedy pathway) [1,11], but a second pathway via N-methylation of phosphatidylethanolamine has also been described [12]. The data presented here demonstrate that the phospholipid analogue, hexadecylphosphocholine, inhibits phosphatidylcholine biosynthesis via the CDP-

Table I

Effect of hexadecylphosphocholine on the lipid composition of MDCK-cells

MDCK-cells grown to confluence were incubated with 50 μM hexadecylphosphocholine for 24 h. For control experiments no supplements were added. The cells were mechanically harvested and the lipids were extracted according the method of Bligh and Dyer [17] as described in section 2. The lipids were separated by HPTLC, stained with cupric sulfate reagent and quantified by densitometric videoscanning. The values are given in % of the total lipid extract ($n = 3$). PC, phosphatidylcholine; SM, sphingomyelin; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; CL, cardiolipin; TG, triacylglycerol.

	SM	PC	PS	PI	PE	CL	TG
Control*	5.3±0.6	36.0±0.9	8.6±1.2	5.9±0.7	19.3±0.9	10.8±1.4	14.1±1.4
HePC**	4.9±0.6	29.9±0.2	9.6±1.0	5.3±0.4	24.3±0.9	10.1±1.6	15.9±1.1

Total lipids (100%) were 99.5 ± 9.5 μg/10^6 cells for control cells* and 92.9 ± 5.0 μg/10^6 cells for HePC-treated cells**

choline pathway in MDCK cells. It is known that the rate-limiting step of this pathway is catalyzed by CTP:phosphocholine cytidylyltransferase (EC 2.7.7.15) [1]. The accumulation of radiolabelled phosphocholine in hexadecylphosphocholine-treated cells and the inhibition of the cytidylyltransferase in vitro suggest that the rate-limiting step of phosphatidylcholine biosynthesis is the target of inhibition.

In earlier studies it was demonstrated that cytidylyltransferase activity is regulated by translocation of the inactive cytosolic form to the membrane, where it becomes active. A number of factors have been implicated in this translocation. Diacylglycerol [13], fatty acids [14], lysophosphatidylethanolamine [15] and a protein dephosphorylation reaction [16] will each facilitate association of cytidylyltransferase with membranes. Conversely, lysophosphatidylcholine [17] and sphingosine [10] inhibit the activity of cytidylyltransferase. Since hexadecylphosphocholine is structurally similar to lysophosphatidylcholine it may interfere with the translocation and hence act as a direct inhibitor of the cytidylyltransferase.

On the other hand, hexadecylphosphocholine is an inhibitor of protein kinase C [4,5] and therefore the possibility of an indirect action should be taken into account. In further studies we will address ourselves to the mechanism underlying the described inhibitory effect.

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