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MODULATION OF CTP : PHOSPHOCHOLINE CYTIDYLYLTRANSFERASE TRANSLOCATION BY OLEIC ACID AND THE ANTITUMORAL ALKYLPHOSPHOLIPID IN HL-60 CELLS

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Short time effect of oleate and 1-O-alkyl-2-O-methyl-rac-glycero-3-phosphocholine (AMGPC) on choline incorporation into phosphatidylcholines were studied in HL-60 cells. The non lytic concentration of 50 µM oleate induced a three-fold increase in [^{3}H]choline incorporation into phosphatidylcholine. This stimulation was accompanied by a translocation of the CTP:phosphocholine cytidylyltransferase (EC 2.7.7.15) from cytosol to membranes. By contrast, the ether-lipid AMGPC inhibited [^{3}H]choline incorporation into phosphatidylcholine by 60% at 10 µM. AMGPC had no effect on choline kinase or choline phosphotransferase activities. When AMGPC was added separately to an homogenate, a particulate or a cytosolic fraction, cytidylyltransferase inhibition was observed only in the homogenate. However on particulates recovered from homogenates treated with increasing concentrations of AMGPC, membranous cytidylyltransferase activity decreased dose-dependently. Thus AMGPC had no effect on cytidylyltransferase activity itself but inhibited its translocation from cytosol to membrane. At variance with the well-established positive effect on cytidylyltransferase translocation induced by fatty acids, this is the first demonstration that AMGPC can inhibit cytidylyltransferase translocation in cell-free system.

In all the cells studied so far, phosphatidylcholine synthesis is regulated by CTP:phosphocholine cytidylyltransferase (EC 2.7.7.15) [1,2]. The enzyme is translocated from cytosol (inactive form) to membranes (active form) by various stimuli such as phospholipase C [3,4,5], reversible protein phosphorylation [6] or fatty acids, oleic acid being the most potent agent inducing translocation [1,7,8,9].

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Recently, the modulation of cytidylyltransferase activity by various lipidic structures, i.e. presence of alkyl chain and amino group, was demonstrated on model membrane [10]. The antitumoral ether lipid 1-O-alkyl-2-O-methyl-rac-glycero-3-phosphocholine (AMGPC) disturbs membrane structure [11], modulates protein kinase C activity [12,13] and inhibits Na^+,K^+ ATPase activity [14]. This agent also inhibits choline uptake and incorporation into HL-60 cells [15,16]. However, these various reports dealt with long-term effects of AMGPC (between 4 to 24 h). In addition, only a few investigations on the effect of ether-lipids on the enzymes of the de novo pathway of phospholipid synthesis have been performed.

In the present work, we have studied the differential effect on phosphatidylcholine synthesis of short term exposure (from 5 to 30 min) of HL-60 cells to AMGPC and oleate. We show that non lytic concentrations of oleate and AMGPC displayed opposite effects on cytidylyltransferase translocation.

MATERIALS AND METHODS

[Methyl-^{3}H]choline chloride (81.8 Ci/mmol), phosphoryl[methyl-^{14}C]choline (56 mCi/mmol) and cytidine 5'-diphospho[methyl-^{14}C]choline (50 Ci/mmol) were purchased from The Radiochemical Center, Amersham, U.K.. Alkylmethoxy-GPC (1-O-alkyl-2-O-methyl-sn-glycero-3-phosphocholine), oleic acid, choline, phosphorylcholine, cytidine diphosphocholine (CDP-choline), cytidine 5' triphosphate (CTP) and adenosine 5' triphosphate were purchased from Sigma, Saint-Louis, MO, U.S.A.. RPMI 1640 culture medium and glutamine were obtained from Intermed, Noisy-Le-Grand, France ; streptomycine and penicilline from Bio Merieux, Lyon, France ; foetal calf serum from Boehringer, Mannheim, Germany and fungizone from Gibco, Grand Island, U.S.A.. Silicagel G plates were purchased from Merck, Darmstadt, Germany ; Picofluor 15 and Instafluor scintillation cocktails from Packard Instruments, U.S.A.

Stock solution of oleate was prepared by dissolving oleic acid in 0.12 M KOH/95% ethanol and stored at -20°C. Immediately prior use, ethanol was evaporated and oleate dissolved in RPMI 1640/6 mM EGTA by vigourous shaking and sonication. AMGPC was solubilized in 0.15 M NaCl with 0.3% (w/v) bovine serum albumine by mixing.

Cell culture: HL-60 promyelocytic leukemia cells from the American Type Culture Collection, were maintained at 37°C in humidified 5% CO2/95% air atmosphere. The cells were transferred every 5-7 days to RPMI 1640 medium supplemented with 10% foetal calf serum, penicilline (50 units/ml), streptomycine (25 µg/ml), fungizone (2 µg/ml) and glutamine (4 mM).

[^{3}H]choline incorporation into phosphatidylcholine in whole cells: HL-60 cells were washed twice with RPMI/Hepes 40

mM, pH 7.4. The cell suspension (4.10^6 cells/ml) was incubated at 37°C under gentle shaking with 1 µCi/ml [^{3}H]choline (140 µCi/mmol), and AMGPC or oleate were added. At each incubation time, aliquots of the cell suspension were harvested and centrifuged (9,700 x g for 1 min). The pellet was extracted according to Bligh and Dyer [17] and its radioactivity determined.

Cell-free system experiments: HL-60 cells were washed twice with TKM buffer (25 mM Tris HCl, pH 7.4, 100 mM KCl, 5 mM $MgCl_2$) and suspended at a concentration of 2.10^7 cells/ml in ice-cold lysis buffer (25 mM Tris HCl, pH 7.4, 100 mM KCl, 5 mM $MgCl_2$, 1 mM ATP). Lysis was performed by nitrogen cavitation [18] for 5 min at 60 atm. Homogenate was incubated at 37°C for 30 min with either AMGPC or oleate. Cytosol and particulate were obtained by centrifugation in a TFT 80.2 Kontron rotor (300,000 x g, 5 min).

Enzymes assays: CTP:phosphocholine cytidylyltansferase activity was measured according to Sleight and Kent [19] and Tercé et al. [5] with 200 µg of protein. The 1,2 diacylglycerol cholinephosphotransferase (E.C.2.7.8.2) was measured according to ref.[5] and choline kinase (E.C.2.7.1.32) as described in ref [19]. Water-soluble choline derivatives were separated according to Vance et al.[20].

Miscellaneous: Proteins were measured according to the procedure of Lowry et al [21] in presence of 0.07% sodium dodecyl sulfate with bovin serum albumin as standard. Lactate deshydrogenase activity [22] was measured as a control of cellular lysis. Total lysis was obtained by treatment of cells with 0.05% (v/v) Triton X100.

Data presented are representative of two to three experiments performed under identical conditions.

RESULTS

I - Stimulation of [^{3}H]choline incorporation into PC and cytidylyltransferase translocation by oleate in HL-60 cells.

Stimulation of [^{3}H]choline incorporation into phosphatidylcholine exhibited a sharp dependence towards oleate concentration (Fig.1). Oleate at 40-50 µM provoked a 3 to 4-fold enhancement in phosphatidylcholine labelling, in the absence of cell lysis (Fig.1). An oleate concentration of 60 µM provoked a noticeable increase in cell lysis and stimulation of [^{3}H]choline incorporation was abolished. Therefore further experiments were conducted at non lytic oleate concentrations.

The effect of oleate was also investigated on the cellular distribution of cytidyltransferase. In non-treated cells, a high amount of the enzyme was soluble since 89 ± 1.8 % of the activity (measured in presence of lipids) was found

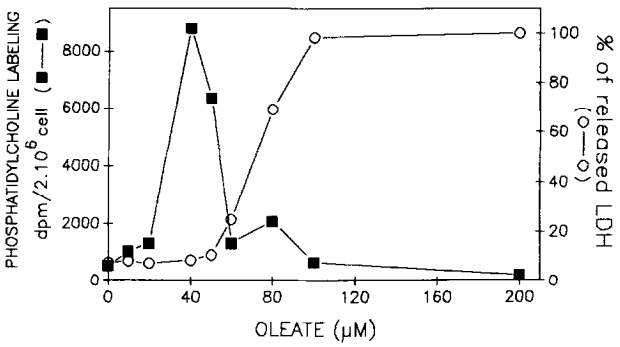


Figure 1 . **Concentration dependent stimulation by oleate of [^{3}H]choline incorporation into phosphatidylcholine.** HL-60 cells were incubated with [^{3}H]choline (1 μCi/ml) and various concentrations of oleate for 30 min at 37°C. The reaction was stopped by centrifugation and radioactivity in phosphatidylcholine was determined (■). Cell integrity was monitored by release of lactate deshydrogenase (○)

in the cytosol. When an homogenate was incubated for various times with 50 μM oleate, we observed the translocation of cytidylyltransferase from cytosol to membranes, which was achieved within 20 min (Fig.2). This enzyme redistribution was

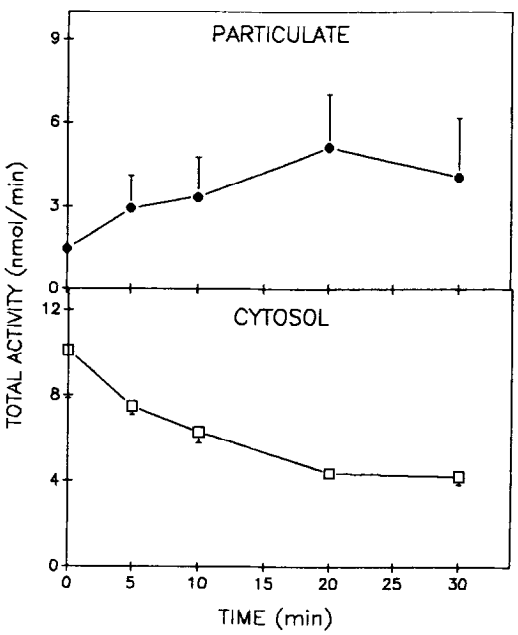


Figure 2 . **Time dependent translocation of cytidylyltransferase in presence of oleate.** Homogenate was incubated with 50 μM oleate at 37°C. At each incubation time, cytosol and particulate fractions were separated and assayed for cytidylyltransferase activity. Data represent means ± SEM of total activity from 10^8 cells.

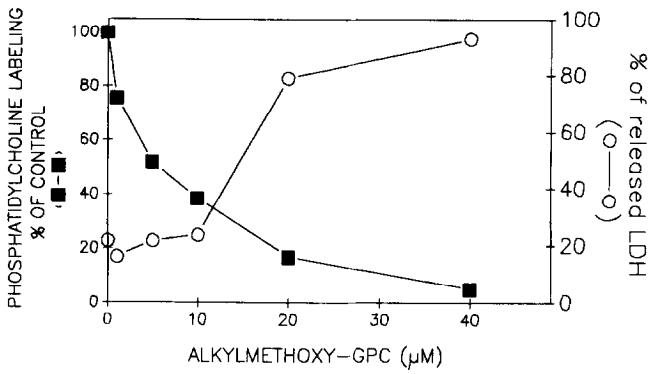


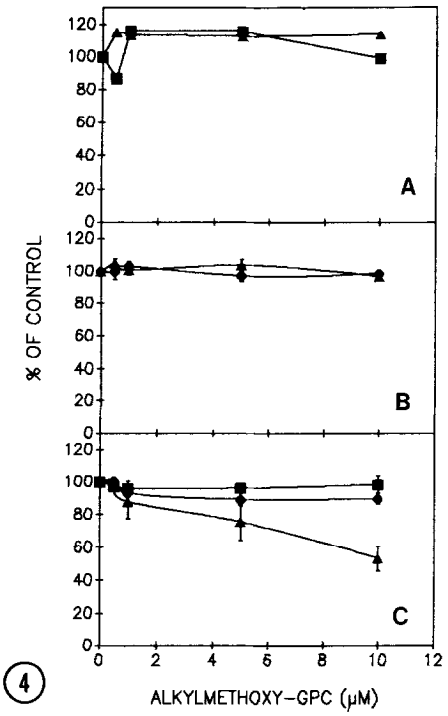
Figure 3. **Effect of AMGPC on [^{3}H]choline incorporation into phosphatidylcholine:** HL-60 cells were incubated with 1 μCi/ml [^{3}H]choline for 30 min in presence of various concentrations of AMGPC. Radioactivity in phosphatidylcholine was determined (■) comparatively to the release of lactate deshydrogenase (○).

concentration-dependent in the range of non-lytic concentrations of oleate (not shown).

II - Inhibition of [^{3}H]choline incorporation and cytidylyltransferase translocation by AMGPC .

The effect of short time exposure to AMGPC was investigated. The compound induced a dramatic concentration-dependent inhibition of [^{3}H]choline incorporation into phosphatidylcholine (Fig. 3). In the range of non-lytic concentrations, 10 μM AMGPC induced a 60% inhibition of phosphatidylcholine labeling (Fig. 3).

This inhibition of choline incorporation could be due to a direct effect of AMGPC on one or more of the enzymes involved in the de novo pathway for phosphatidylcholine synthesis. Therefore, the various enzyme activities involved were measured in the presence of AMGPC, up to 10 μM, in the subcellular compartments where they are usualy located (Fig. 4). We observed no effect of AMGPC on the choline kinase activity measured in the homogenate (0.85 nmol x min^{-1} x mg^{-1}) or cytosolic fraction (0.94 nmol x min^{-1} x mg^{-1}) (fig. 4A). Similarly, the choline phosphotransferase measured in the homogenate (0.06 nmol x min^{-1} x mg^{-1}) or particulate fraction (0.22 nmol x min^{-1} x mg^{-1}) (Fig. 4B) was not affected by AMGPC. However, a concentration-dependent inhibition of the cytidylyltransferase in the homogenate was observed (Fig. 4C).



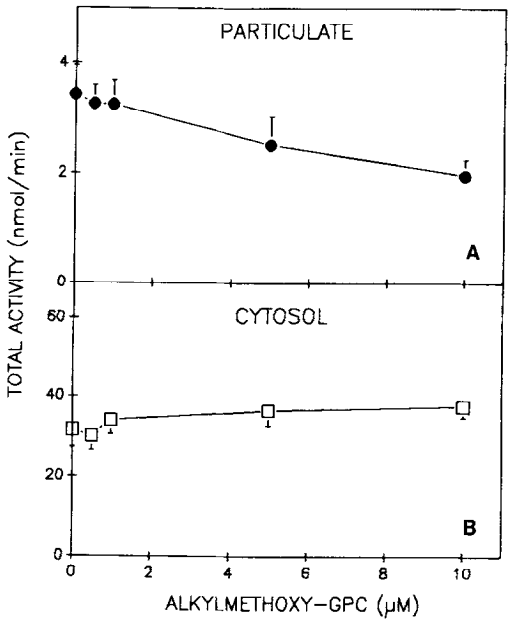


Figure 4 . **Effect of AMGPC on the enzyme activities of phosphatidylcholine de novo synthesis.** Homogenate, cytosol and particulate fractions were obtained as described in Material and Methods. The enzyme activities were measured in presence of various concentration of AMGPC (▲ : homogenate, ■ : cytosol fraction, ● : particulate fraction).
A : choline kinase, B : choline phosphotransferase, C: cytidylyltransferase. Results (means ± SEM) are expressed in percentages of control activity measured in the absence of AMGPC.

Figure 5 . **Effect of AMGPC on membrane-bound cytidylyltransferase activity.** HL-60 cell homogenates were incubated with various concentrations of AMGPC for 30 min at 37°C. Particulate and cytosol fractions were then separated and tested for cytidylyltransferase activity, measured in presence of lipids for cytosol. Data represent means ± SEM of total activity from 10^8 cells.

The compound at 10 μM induced a 50 % inhibition of the specific activity (1.79 ± 0.14 versus 0.95 ± 0.21 nmol x min^{-1} x mg^{-1} in control and treated homogenates, respectively). However this inhibition was not found in cytosol and particulate fraction checked separately (Fig.4C). Therefore, AMGPC had no direct inhibitory effect on both cytosolic and particulate cytidylyltransferase activity but instead inhibited its expression in the homogenate.

To further investigate this observation, homogenates were incubated with AMGPC (0.5 to 10 μM) for 30 min. Subcellular

fractions were then rapidly separated with a high-speed rotor (see Material and Methods), and cytidylyltransferase activity determined in particulate and cytosolic fractions. Under these conditions, the total activity in control particulate fraction (no AMGPC) was higher (Fig 5) than in the experiment depicted in Fig 4, when particulate was first prepared prior to cytidylyltransferase determination (3.43 ± 0.52 nmol x $min.^{-1}$ and 2.57 ± 0.32 nmol x $min.^{-1}$, respectively). Our results indicated an increase in membranous activity of the cytidylyltransferase during incubation of total homogenate. However this membrane-bound activity decreased dose-dependently with increasing concentrations of AMGPC (fig.5A). Because of the high soluble cytidylyltransferase activity, the decrease of particulate activity provoked only a slight increase in the cytosolic one (from 31.71 ± 4.25 nmol x min^{-1} in control to 37.62 ± 2.85 nmol x min^{-1} with 10μM AMGPC; Fig.5B).

We concluded that AMGPC effect in the homogenate (Fig 4C) was to release the membrane-associated cytidylyltransferase, whereas the compound had by itself no inhibitory effect on the enzymatic reaction, taking place either on the particulate or the cytosolic compartments (Fig 4C). We also concluded that the effect of non-lytic AMGPC concentrations in vivo (Fig.3) was, at least in part, to diminish the amount of membrane-bound cytidylyltransferase, with subsequent decrease in [3H]choline incorporation.

Therefore AMGPC released cytidylyltransferase from membranes, whereas oleic acid augmented the membrane-associated form of the enzyme.

DISCUSSION

Enzyme translocation within the cell has become a general process to regulate metabolism in many cells. For example, it is well known that protein kinase C is translocated upon cell activation [23] as well as 5 lipoxygenase [24] or cytidylyltransferase [1]. The latter regulates de novo phosphatidylcholine synthesis in all mammalian cell studied so far. However, only a few studies of this pathway have been performed in HL-60 cells [15,16]. Cytidylyltransferase in this

cell model is also efficiently translocated from cytosol to membranes with oleic acid (Fig. 2), but with a 10-fold lower fatty acid concentration (50 μM, Fig. 1) than in many other cells [25,26], HL-60 cells displaying a higher suseptibility to lysis by oleate dispersions. This translocation process has been shown to depend upon the membrane-bound oleic acid [9].

Similarly, AMGPC has been evidenced to bind to membranes and to be rapidly distributed within the cell [27]. Most of the studies performed with this compound have been conducted during lond term incubations (from 4 to 24 h) and without a careful monitoring of cell lysis. Interestingly, a non-lytic concentration of 10 μM induced a 60% decrease in [^{3}H]choline incorporation into phosphatidylcholine (Fig. 3). The effect of similar AMGPC concentrations in [^{3}H]choline uptake [15] would account after 4 h for only 35 % in the decrease of phosphatidylcholine labelling. Although not checked in the present study, choline content has not been shown to be rate limiting for phosphatidylcholine synthesis in mammalian cells [1,5]. This prompted us to analyze AMGPC effect on the enzymes involved in the de novo pathway. Only cytidylyltransferase in the homogenate is affected by the presence of AMGPC. (Fig. 4), whereas this compound is by itself not inhibitory of the enzyme activity. Incubation of the homogenate increases the amount of membrane-bound cytidylyltransferase, whereas AMGPC reverses the effect (Fig. 5). Consequently, the inhibition of phosphatidylcholine labelling shown in Fig. 3 might also result from interference of AMGPC with the basal amount of membrane-bound cytidylyltransferase in the cell. This latter effect could vary according to the stage of cell differentiation, since differentiated HL-60 cells bind 50 % less of AMGPC, as compared to undifferentiated ones [15]. Differential release of membrane-bound cytidylyltransferase by AMGPC could account, in part, for its differential cytotoxic action according to the stage of cell differentiation [15].

Thus AMGPC appears to behave towards cytidylyltransferase like sphingosine towards protein kinase C [28]. In this respect various similarities have been reported between the two enzymes[2].

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