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The Phospholipid Analogue, Hexadecylphosphocholine, Inhibits Protein Kinase C *in vitro* and Antagonises Phorbol Ester-stimulated Cell Proliferation

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The antineoplastic agent, hexadecylphosphocholine, a phospholipid analogue, inhibited phosphatidylserine-activated protein kinase C *in vitro* at concentrations higher than 40 μmol/l. The half-inhibitory concentration (IC_{50}) was 62 μmol/l. Another alkylphosphocholine, dodecylphosphocholine, did not have an inhibitory effect on protein kinase C. At the same concentrations, hexadecylphosphocholine antagonised the phorbol ester-stimulated proliferation of Madin–Darby canine kidney cells whereas dodecylphosphocholine had no effect. In addition, phorbol ester-induced morphological changes of these epithelial cells were antagonised by hexadecylphosphocholine. Both effects of hexadecylphosphocholine, the inhibition of protein kinase C and the antagonisation of the altered cell morphology induced by phorbol ester, were comparable to those observed after treatment with sphingosine, a known protein kinase C inhibitor. We conclude that one possible mechanism of the antineoplatic action of hexadecylphosphocholine is mediated by inhibition of protein kinase C.
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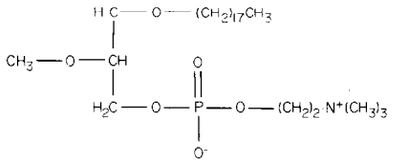
INTRODUCTION

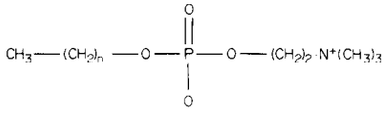
SEVERAL ALKYLLYSOPHOSPHATIDYLCHOLINES have been shown to retard or inhibit the growth of different tumours *in vitro* and *in vivo* [1–5]. It has been proposed that this effect is mediated by enhancement of the cytotoxicity of macrophages [6], by a disturbance of phospholipid metabolism [7] and by a direct influence on membrane-bound enzymes like protein kinase C [8].

The chemical structure of another group of phospholipid analogues, the alkylphosphocholines, is similar to that of the alkyllysophosphatidylcholines (Fig. 1). Therefore, these phospholipid analogues may also be effective antineoplastic agents. Hexadecylphosphocholine, an alkylphosphocholine, has been shown to be a successful anticancer drug against skin metastases of human mammary carcinoma [9]. Its therapeutic activity against mammary tumours was superior to that of cyclophosphamide in animal experiments [10]. Compared with the other known antineoplastic alkyllysophosphatidylcholines, this alkylphosphocholine has the advantage that it is only slowly and poorly metabolised, mainly by cleavage of the choline head group [11].

In the present work, we report that micromolar levels of hexadecylphosphocholine inhibit phosphatidylserine-activated protein kinase C *in vitro* and antagonise phorbol ester (TPA)-stimulated cell proliferation and alterations of cell morphology in Madin–Darby canine kidney (MDCK) cells. In contrast, we demonstrate that another alkylphosphocholine, dodecylphosphocholine, does not have an inhibitory effect on protein kinase C or cell proliferation. Therefore, we regard dodecylphosphocholine as a useful negative control substance in experiments dealing with the effect of hexadecylphosphocholine.



1-Octadecyl-2-methyl-glycero-3-phosphocholine (ET-18-OCH_3)



n = 15: hexadecylphosphocholine (HePC)

n = 11: dodecylphosphocholine (DoPC)

Fig. 1. Structure of hexadecylphosphocholine, dodecylphosphocholine and 1-octadecyl-2-methyl-glycero-3-phosphocholine.

MATERIALS AND METHODS

Materials

Hexadecylphosphocholine was a gift from Dr Engel (Asta Pharma, Frankfurt/Main, Germany), Dodecylphosphocholine was prepared by J. Mulzer and M. Mickeleit, Institut für Organische Chemie, Freie Universität Berlin. [γ-^{32}P]ATP (111 TBq/μmol) was from New England Nuclear. Histone III S, sphingosine and 12-O-tetradecanoyl-phorbol-13-acetate were from Sigma. P81 filter was purchased from Whatman (Maidstone,UK).

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Protein kinase C assay

Protein kinase C was purified from bovine brain by affinity chromatography on protamine agarose [12]. Protein kinase C activity was determined in a total volume of 50 μl in a buffer system consisting of 20 mmol/l triethanolamine (pH 7.4), 4 mmol/l Mg acetate, 0.1 mmol/l $CaCl_2$, 50 mmol/l mercaptoethanol, 20 μg histone III S, 0.5 μg phosphatidylserine (PS) and 20 μmol/l ATP supplemented with [γ-^{32}P] ATP (final radioactivity 2×10^5 cpm). Before addition to the assay mixture, hexadecylphosphocholine and dodecylphosphocholine were diluted to the appropriate concentration in 20 mmol/l triethanolamine (pH 7.4) by brief sonication. For control the same procedure was performed without the alkylphosphocholines. The reaction was started by addition of the enzyme (10–20 ng). After incubation at 30°C for 1 min, 40 μl of the reaction mixture was applied to a Whatman P81 filter. The filter was washed three times with 0.05% phosphoric acid. Radioactivity was determined by measuring Cerenkov radiation in water.

Cell culture

MDCK cells were grown in plastic culture dishes (Nunc, Denmark) containing Dulbecco's minimal essential medium supplemented with 10% calf serum, 0.56 g/l L-glutamine, 100 000 IU penicillin and 0.1 g/l streptomycin. Media and culture reagents were obtained from Gibco, penicillin and streptomycin from Boehringer (Mannheim, Germany). Confluent cells were subcultured every 3 days after detaching the cells with a trypsin/EDTA solution. For experimental purposes, cells were used on the third day of culture. Cytotoxicity of hexadecylphosphocholine to MDCK cells was estimated according to Culvenor *et al.* [13] by measurement of the alkaline phosphatase activity of the cells.

Cell proliferation assay

Cell proliferation was measured by determination of the cell number according to the method of Gilles *et al.* [14]. MDCK-cells (1×10^3 cells per well) were incubated with medium containing additionally either 7.5 nmol/l TPA, 100 μmole hexadecylphosphocholine or 100 μmol/l dodecylphosphocholine, or 7.5 nmol/l TPA plus 100 μmol/l hexadecylphosphocholine or 7.5 nmol/l TPA plus 100 μmol/l dodecylphosphocholine. After 6 h the proliferation rate was determined by the crystal violet assay as described and compared with that of control cells ($n = 6$). Cells were fixed by treatment with 1% glutaraldehyde for 15 min, stained for 30 min in 0.1% crystal violet solution, then washed with deionized water for 15 min. Crystal violet absorbed by cell nuclei was solubilized with 0.2% Triton X-100 and the amount released was quantitated photometrically at 590 nm.

RESULTS

Hexadecylphosphocholine shows an ambiguous effect on the activity of protein kinase C *in vitro* in an assay using phosphatidylserine as activator. At high concentrations (> 40 μmol/l), hexadecylphosphocholine inhibits the enzyme. Surprisingly, hexadecylphosphocholine stimuates protein kinase C slightly at concentrations below 30 μmol/l. From these data a half-inhibitory concentration (IC_{50}) of 62 μmol/l was calculated. Dodecylphosphocholine shows no inhibitory effect on the activity of protein kinase C, but stimuates it slightly, maximal stimulation occuring at a dodecylphosphocholine concentration of 100 μmol/l (Fig. 2).

In order to perform the experiments at an alkylphosphocholine concentration that did not affect cell viability, it was necessary to determine the cytotoxicity of these lipid analogues on MDCK epithelial cells. Up to a concentration of 100 μmol/l, hexadecylphosphocholine did not cause any decrease of alkaline phosphatase activity in the culture medium, indicating that the cells are still viable under these conditions (data not shown). Morphological alterations like rounding off and detachment of cells only occurred at concentrations higher than 1 mmol/l.

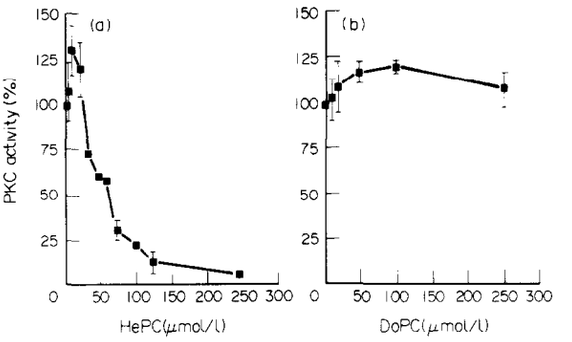

Fig. 2. Dose-dependent influence of hexadecylphosphocholine and dodecylphosphocholine on PS-activated protein kinase C *in vitro*. The number of experiments at each point were between 4 and 8.

Furthermore, the influence of hexadecylphosphocholine and dodecylphosphocholine on cell proliferation of TPA-stimulated and non-stimulated cells was examined. It was shown that hexadecylphosphocholine (100 μmol/l) inhibited the cell proliferation by about 60% and antagonised the TPA-induced cell proliferation of MDCK cells (Fig. 3). The addition of hexadecylphosphocholine (100 μmol/l) plus TPA (7.5 nmol/l) to MDCK-cells decreased cell proliferation by about 20% compared with the control experiment. In contrast, dodecylphosphocholine (100 μmol/l) had no effect on cell proliferation and did not antagonise TPA-induced cell proliferation. Moreover, the morphological alterations induced by TPA (5 nmol/l) were only antagonised by hexadecylphosphocholine (100 μmol/l) and not by dodecylphosphocholine (100 μmol/l) (Fig. 4). TPA changed the epithelial growth behaviour of MDCK cells (Fig. 4a) to a fibroblastic type of growth (Fig. 4b), while MDCK cells incubated in the presence of TPA plus hexadecylphosphocholine showed the normal epithelial morphology (Fig. 4c). Dodecyl-

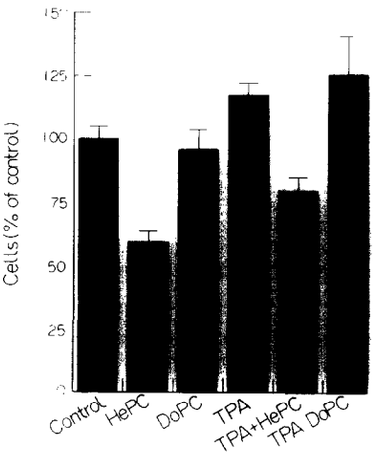

Fig. 3. Influence of hexadecylphosphocholine (HePc), dodecylphosphocholine (DoPc) and TPA on MDCK cell proliferation.

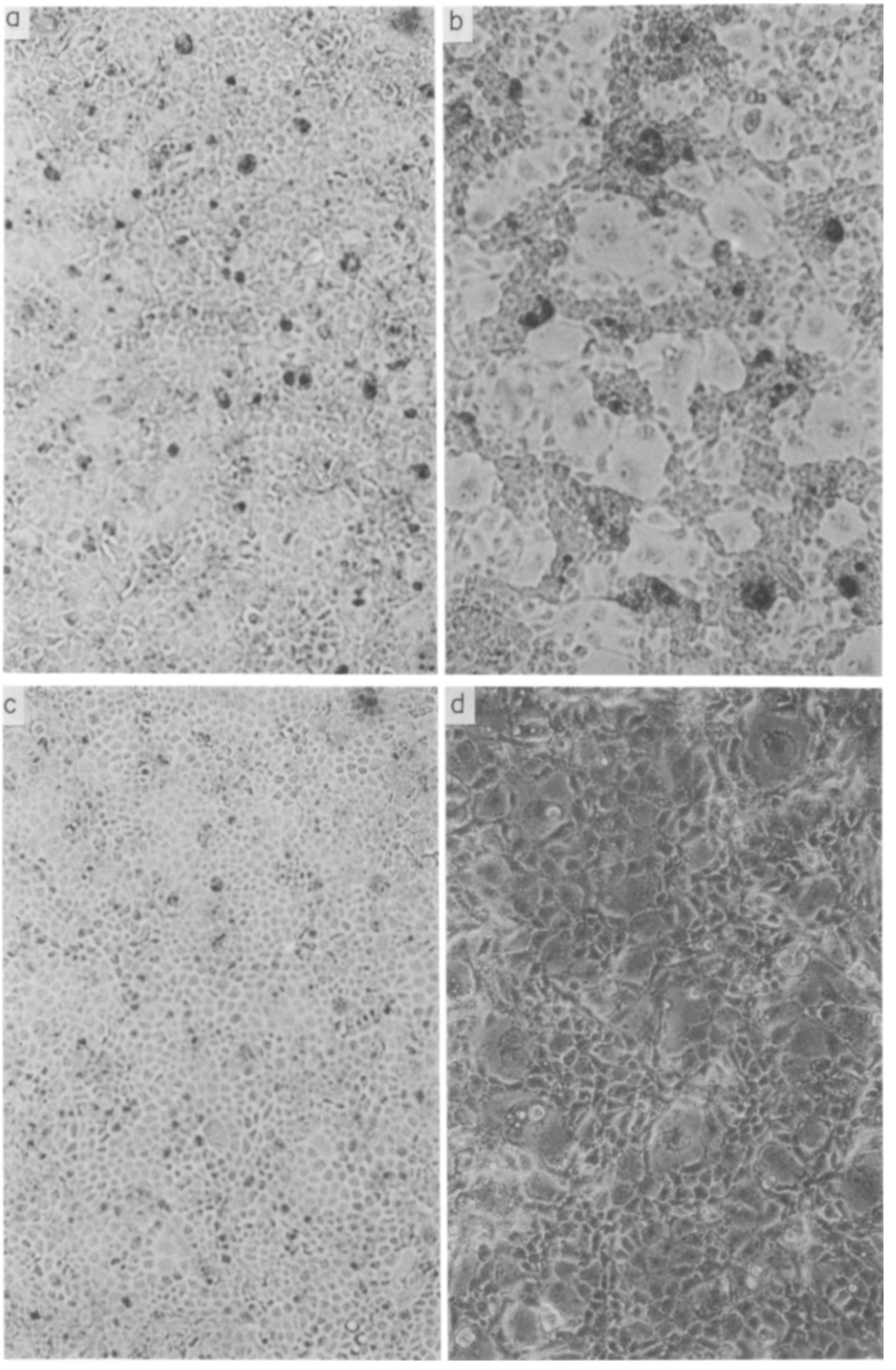

Fig. 4. Morpholgical alteration of MDCK cells treated with TPA. TPA plus dodecylphosphocholine or TPA plus hexadecylphosphocholine. MDCK cells were incubated for 12 h in cell culture medium containing no additional supplements (a), 5 nmol/l TPA (b), 5 nmol/l TPA plus 100 μmol/l hexadecylphosphocholine (c),or 5 nmol/l TPA plus 100 μmol/l dodecylphosphocholine (d). Magnification × 125.

phosphocholine did not alter the cell morphology (not shown) and did not antagonise TPA-induced morphological changes (Fig. 4d).

The inhibition of cell proliferation by hexadecylphosphocholine as well as the induced morphological alterations were similar to those caused by sphingosine (5 μmol/l), a known inhibitor of protein kinase C (data not shown).

DISCUSSION

Recently it was shown that the phospholipid analogue, hexadecylphosphocholine, can be used as an antineoplatic drug, especially for the treatment of cutaneous metastases of breast cancer [9]. In the present study, it was demonstrated that the phosphatidylserine-activated protein kinase C is effectively inhibited by hexadecylphosphocholine *in vitro*. This is in accord-

ance with findings of Zheng *et al.* [15] and Überall *et al.* [16]. Earlier studies showed that lysophosphatidylcholine, which is structurally similar to hexadecylphosphocholine, influences protein kinase C in the same manner [17]. Therefore, the effect of another alkylphosphocholine, dodecylphosphocholine, was measured. It is interesting that this very similar analogue did not inhibit protein kinase C, indicating that the alkyl moiety must have a certain length in order for the compound to display an inhibitory effect.

Protein kinase C is a component of transmembrane signalling systems [18]. Agonist stimulated hydrolysis of phospholipids produces the second messenger, diacylglycerol (DAG), which stimulates the movement of protein kinase C to the cell membrane. Tumour-promoting phorbol esters like TPA substitute for DAG as activator, and it has been shown that this activation leads to an increased cell proliferation [19]. Our experimental model shows the inhibitory effect of hexadecylphosphocholine on TPA-stimulated cell proliferation.

In addition, the TPA-induced stimulation of cell proliferation and its antagonisation by hexadecylphosphocholine were paralleled by typical morphological alterations. The typical epithelial growth behaviour of MDCK cells was changed to a fibroblastic type of growth by TPA treatment, whereas MDCK cells incubated in the presence of TPA plus hexadecylphosphocholine showed the normal epithelial morphology. These findings support our hypothesis that hexadecylphosphocholine inhibits cell proliferation via protein kinase C inhibition. Therefore, we repeated the cell proliferation experiments with a known protein kinase C inhibitor, sphingosine. The results obtained after by treatment with sphingosine (5 μmol/l) were similar to those obtained after treatment with hexadecylphosphocholine in the same experimental model of TPA stimulated MDCK cells.

Dodecylphosphocholine, another alkylphosphocholine, did not show any effects like hexadecylphosphocholine. Therefore, it should be regarded as a useful negative control substance with similar physical properties in experiments investigating the effect of hexadecylphosphocholine.

In conclusion, the alkylphosphocholine, hexadecylphosphocholine, alters the growth and cellular morphology of TPA-stimulated MDCK-cells. The effect is paralleled with its inhibitory effect on protein kinase C *in vitro*. Therefore, we propose that one possible mechanism of the antineoplastic action of hexadecylphosphocholine may be an inhibitory effect on protein kinase C, and not only a cytotoxic effect of hexadecylphosphocholine and its metabolites as suggested recently [20]. On the other hand, it should be taken into account that besides this effect on protein kinase C activity *in vitro*, hexadecylphosphocholine was shown to inhibit further membrane-associated enzymes, e.g. Na K ATPase *in vitro* [15] and CTP:phosphocholine cytidylyltransferase *in vitro* and *in vivo* (unpublished results). Consequently, we suggest that the antineoplastic action of hexadecylphosphocholine has more than one target in the cell metabolism.

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