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Effect of palmitoylcarnitine on the cellular differentiation, proliferation and protein kinase C activity in neuroblastoma nb-2a cells

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Abstract. Palmitoylcarnitine is synthesized through the action of palmitoylcarnitine transferase I - an enzyme specifically inhibited by etomoxir. An increase of the intracellular content of palmitoylcarnitine in neuroblastoma NB-2a cells after administration of carnitine was correlated with an inhibition of cell proliferation and a concomitant promotion of differentiation processes. The activity of protein kinase C was measured *in vivo*, with cells permeabilized through the use of streptolysin O and a peptide substrate. Palmitoylcarnitine inhibited the phorbol ester stimulated reaction of the peptide phosphorylation in a concentration dependent way. The degree of protein kinase C inhibition was correlated with intracellular increase of the palmitoylcarnitine content, pointing to this compound as a natural modulator of protein kinase C activity.

Key words: palmitoylcarnitine, protein kinase C, neuroblastoma, differentiation, proliferation, etomoxir

INTRODUCTION

Calcium and phospholipid dependent protein kinase C (PKC), playing a central role in the regulation of many cellular processes in receptor mediated signal transduction, is involved in a variety of cellular responses. With a specificity for serine and threonine, PKC has been linked to receptor activation through the second messenger - diacylglycerol (Nishizuka 1984, 1986, Wilkinson and Hallam 1992). Phosphorylation of various proteins by PKC influence the function of many cell surface receptors, processes of secretion, modulation of ion channel activities, cellular metabolism and gene expression (Nishizuka 1984, 1986, Jaken 1992, Knox and Kaczmarek 1992, Berridge 1993). All these changes can have an important effect on the control of cell growth and division on the one hand, and cell differentiation on the other (Clemens et al. 1992). Known lipid activators of PKC, apart of diacylglycerol, include Ca/phosphatidylserine, unsaturated fatty acids, arachidonic acid and phosphatidylinositol-4,5-diphosphate (Bell and Burns 1991). Tumor promoting phorbol esters substitute diacylglycerol as activators of PKC and promote the translocation of PKC to plasma membrane where the rapid limited proteolysis of PKC occurs *via* the action of calpain (Kishimoto et al. 1989).

One of the possible modulators of PKC activity could be palmitoylcarnitine. This long-chain carnitine ester was reported to decrease the activity of PKC in heart (Katoh et al. 1981, 1983), brain (Turner et al. 1982, Nakadate and Blumberg 1987) and epiderm (Brockenbrough and Korc 1987). It is supposed that this inhibition leads to other biological effects like the inhibition of epidermal growth factor association with cells (Brockenbrough and Korc 1987), blockage of gap junctional intercellular communication (Oh et al. 1988) or inhibition of melanoma cell proliferation (Vescovi et al. 1988). In these cells palmitoylcarnitine was described to modulate PKC activity in a complex manner, following a sigmoidal dependence on palmitoylcarnitine concentration (Vescovi et al. 1988).

The long chain acylcarnitines are synthesized from the acylCoA forms of fatty acids due to the activity of palmitoylcarnitine transferases, located either in peroxisomes or in mitochondria (Bremer 1983, Bieber 1988, Guzman and Geelen 1992). The enzymes in mitochondria, together with the carnitine carrier from the inner mitochondrial membrane, are responsible for transfer of acyl compounds from the cytosol to the mitochondrial matrix where fatty acids are further metabolized. This involvement of carnitine in delivering substrates for β-oxidation has been described for peripheral tissues (Bremer 1983, Bieber 1988). In neurones, where the level of fatty acid β-oxidation is low (Warshaw and Terry 1976, Nehlig and Pereira de Vasconcelos 1993), another physiological role was proposed for the mitochondrial carnitine carrier due to its different substrate specificity and properties (Kamińska et al. 1995). It has been postulated that this transporter can be responsible for the export of acetyl moieties for the acetylcholine synthesis (Wawrzeńczyk et al. 1994, 1995a). Neurones also accumulate long-chain acylcarnitines, which in brain (Bresolin et al. 1982) and isolated rat cerebral cortex neurones (Wawrzeńczyk et al. 1995a, 1995b) do not exceed 20% of all carnitine derivatives. Unexpectedly, a very high content of long-chain acyl carnitines was detected in neuroblastoma cells (Nałęcz et al. 1995). Since this is a quickly proliferating cell line of transformed neurones it seemed interesting to analyse in more detail the effect of palmitoylcarnitine on the activity of PKC in these cells. For this purpose, various experimental conditions were applied to modify the intracellular content of palmitoylcarnitine and to correlate the level of this compound with the exerted effect on PKC activity.

METHODS

Materials

The mouse neuroblastoma NB-2a cell line derived from the C 1300 tumor (Augusti-Tocco and Sato 1969) was kindly supplied by Prof. A. Azzi from the Institute for Biochemistry and Molecular Biology, University of Berne, Switzerland. Tissue culture plastics were from Corning. Minimum essential medium with Earles salts (EMEM), foetal bovine serum (FBS), antibiotic/antimycotic mixture and PKC peptide substrate {[ser^{25}]PKC(19-31)} were from Gibco.

L-[methyl-^{3}H]carnitine and [methyl-^{3}H]thymidine were from Amersham, [γ-^{32}P]ATP was delivered by NEN (Świerk, Poland). L-Acylcarnitines of various chain length were from Serva, L-carnitine was provided by Fluka. Etomoxir (sodium 2-[6-(4-chlorophenoxy)hexyl]oxirane-2-carboxylate dihydrate) was a generous gift of Byk Gulden Lomberg Chemische Fabrik GmbH Konstanz, Germany). Streptolysin O (SLO) from *Streptococcus pyogenes* and phorbol 12,13-dibutyrate (PDBu) were from Sigma. Filter paper P-81 was

from Whatman, aluminium silica gel 60 (sheets) were from Merck. Polystyrol foil from Conform (Aalst, Belgium) was a generous gift of Prof. H.-J. Galla from the Institute for Biochemistry, Münster, Germany. Ultima Gold from Packard (Groningen, The Netherlands) was used as a scintillation cocktail. All other reagents were of analytical grade.

A stock solution of 1 mM palmitoylcarnitine in 0.5 mM bovine serum albumine (BSA) was prepared as given by Brockenbrough and Korc (1987), with the exception that HEPES buffered Ringer's solution was replaced with PBS, for controls BSA was mixed with ethanol in an analogous way.

Cell culture

Neuroblastoma NB-2a cells were grown in EMEM containing 25 mM bicarbonate, 100 U penicillin/ml, 100 μg streptomycin/ml, 0.25 μg fungizone/ml and 10% FBS, pH 7.4. Cells, passaged at 1:3 ratio, were usually seeded into 100 mm plastic culture dishes and grown to confluence at 37°C in a humid atmosphere of 5% CO_2. Culture media were changed every three days.

Estimation of cell proliferation

An influence of any compound on neuroblastoma NB-2a cell proliferation was checked by a direct counting method. Cells were seededed into 24-well tissue culture plates at the density 10^4 cells/well, when attached, they were deprived of serum for 24 h and next 5% FBS, 95% EMEM was added, supplemented with the compounds indicated in the figure legends. Cells were collected at the indicated times by trypsinization. The reaction was stopped by addition of FBS containing medium. The cells were spun down at 1,000 xg for 10 min and suspended subsequently in PBS. The amount of cells were counted in a hemocytometer with use of 0.5% trypan blue for the cell viability test.

The synthesis of DNA was determined by incorporation of [methyl-^{3}H]thymidine. Cells were deprived of serum and subsequenty restimulated by an increase of the FBS concentration to 5%. The compounds studied were added together with FBS. The solution of [methyl-^{3}H]thymidine (8.6 Ci/mmol) was added after 24 h and the cells were harvested after 48 h from FBS supplementation. In order to collect the cells the layers were washed twice with Hank's saline supplemented with 10 mg/ml glucose and 1 mg/ml BSA, fixed for 20 min with ice-cold 5% trichloroacetic acid (TCA), briefly washed with ice-cold absolute ethanol and solubilized with warm (40°C) solution of 1% sodium dodecyl sulfate (SDS), 0.1 M NaOH and 2% Na_2CO_3. Aliquots were taken for radioactivity counting.

Accumulation of carnitine

Cells were grown in the absence or presence of etomoxir for 48 h, the culture medium was subsequently removed. Next the cells were washed once with PBS and incubated for 2 h at 37°C in PBS supplemented with 5 mM glucose in the presence of the indicated concentration of L-[methyl-^{3}H]carnitine (15 Ci/mol). In order to terminate the reaction, the monolayer was washed 5 times on the Petri dish with PBS and the cells collected with a cell lifter and spun down at 5,800 x g for 10 min.

Separation of free carnitine and its acyl derivatives was performed as described by Huth et al. (1981). The cellular pellet was treated with 10% TCA and homogenized by passing through a 25 G (0.45 x 16 mm) needle. After keeping the sample for 30 min on ice, the material was spun down for 20 min at 20,000 x g. The pellets were subsequently washed twice with ice cold TCA and parallel samples were either extracted with ethanol for TLC analysis or dissolved in 1% SDS, 0.1 M NaOH, 2% Na_2CO_3 at 40°C with aliquots taken for radioactivity counting and protein estimation. TCA from the supernatant was extracted with diethylether, the samples lyophilized, dissolved in ethanol/water (3:1 v/v) and subjected to TLC analysis as described by Huth et al. (1981). The samples were supplemented with carnitine, acetylcarnitine, propionylcarnitine, hexanoylcarnitine, octanoylcarnitine, lauroylcarnitine, palmitoylcarnitine and stearoylcarnitine (2 mg/ml each) as the carriers. Carnitine derivatives were separated by TLC on silica gel in the following solvent system: methanol/chloroform/water/ammonia/formic acid (110:100:20:15:5) (Huth et al. 1981). The position of carnitine derivatives was defined by comparison with the respective markers run in parallel and visualized by iodine and the amount of respective derivatives was quantified by scratching off 0.5 cm strips of the silica gel and counting the radioactivity in the presence of a scintillation cocktail. The results were normalized to the total radioactivity of the pellet and supernatant estimated before further procedures.

PKC activity assay

The method was adopted from Alexander et al. (1990). Cells were removed from Petri dishes by trypsinization, collected in 10% FBS, EMEM and spun down at 1,000 x g for 10 min. They were resuspended in buffer ICB, containing 5.16 mM $MgCl_2$, 94 mM KCl, 12.5 mM Pipes, 12.5 mM EGTA, 8.17 mM $CaCl_2$, pH 7.4 (Alexander et al. 1990). 200 µl portions, containing 200 µg of cellular protein were taken per assay. In order to introduce ATP and the peptide substrate to the cells, the plasma membrane of NB-2a cells was permeabilized with SLO, as the pores formed by this toxin are large enough to let even antibodies into the cells (Ahnert-Hilger et al. 1989). SLO from *Streptococcus pyogenes* was dissolved in water at a concentration 6U per µl, and further diluted 1:100 with water in the presence of 2 mM DTT (Ahnert-Hilger et al. 1989). In order to find optimal conditions for the permeabilization process, the NB-2a cells were incubated for 25 min with increasing amounts of SLO and the relative content of cells permeable to trypan blue was estimated. The final concentration of 6 U/ml was chosen for further experiments. The phosphorylation reaction was started by the addition of 20 µM peptide substrate and 200 µM [γ-^{32}P]ATP (100 cpm/pmol) and after 12 min was terminated by the addition of 100 µl of 25% (w/v) TCA in 2 M acetic acid. After being kept for 10 min on ice, the samples were spun down (20,000 x g, 10 min) and aliquots from the supernatants were transferred to P-81 ion exchange chromatography paper discs mounted on a Millipore manifold. The discs were washed three times with 2 mM ATP in 30% (v/v) acetic acid containing 1% (v/v) H_3PO_4, removed and washed extensively (3x25 ml per disc) with 30% (v/v) acetic acid containing 1% (v/v) H_3PO_4 by a batch technique. The discs were washed subsequently with ethanol, dried and taken for radioactivity counting.

Preparation of cells for scanning electron microscope imaging

Cells were seeded on polystyrol foil attached to the bottom of wells. Serum was withdrawn for 24 h and subsequently the culture medium was supplemented for the next 48 h with serum without or with compounds indicated in the figure legends. The monolayer was fixed with 2.5% glutaraldehyde buffered with PBS. After washing out glutaraldehyde in the presence of PBS, the cells were further treated for 1 h with 1% osmium tetraoxide in PBS and finally washed once more with PBS. The cells were subsequently dehydrated through a graded series of ethanol and acetone and dried by the CO_2 critical point method. The foil was mounted on a metal stub and coated with carbon and gold. The cells were examined in a JEOL 1200EX electron microscope equipped with an ASID 10 scanning attachment operating at 40 kV.

Protein estimation

The protein concentration was determined by the Lowry procedure (Lowry et al. 1951), modified by the addition of 1% SDS (Nałęcz et al.1986).

RESULTS

Due to the reported effects of palmitoylcarnitine on the functions, morphology and proliferation in different tissues, the studies on a possible influence of palmitoylcarnitine on the growth of neuroblastoma NB-2a

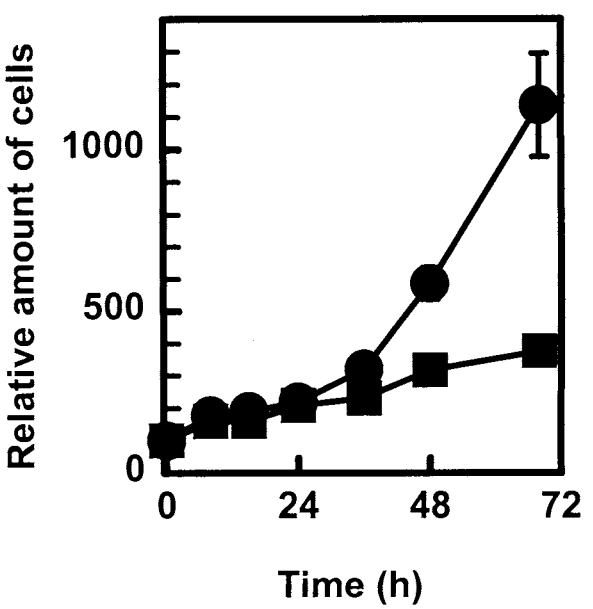

Fig. 1. Effect of palmitoylcarnitine on proliferation of neuroblastoma NB-2a cells. Cells were deprived of serum for 24 h and subsequently grown for the indicated time in the presence of 5% FBS either supplemented with BSA/ethanol (circles) or with 100 µM palmitoylcarnitine in ethanol, bound to BSA (squares), as described in the Methods section. The amount of cells after the indicated time was estimated by direct counting in hemocytometer. The results are means ± SD from 9 independent estimations.

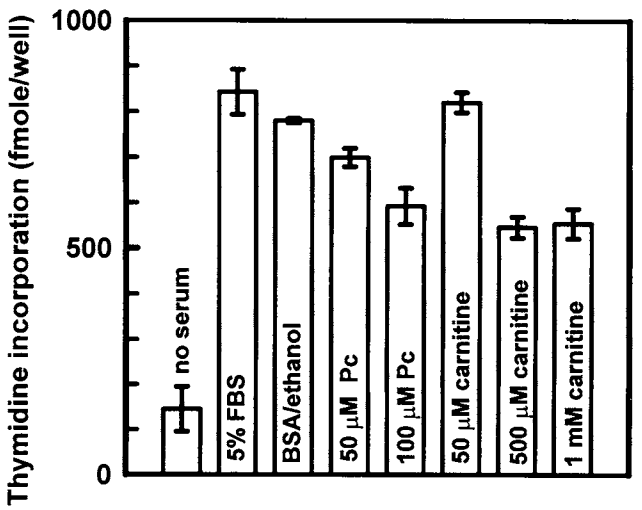

Fig. 2. Effect of palmitoylcarnitine on incorporation of thymidine in neuroblastoma NB-2a cells. After 24 h deprivation of serum, the cells were supplemented for the next 48 h with 5% FBS with either palmitoylcarnitine or carnitine at the concentrations indicated in the graph. Palmitoylcarnitine (Pc) was dissolved in ethanol and coupled to BSA, so the corresponding control (addition of BSA with ethanol) was included. [^{3}H]Thymidine was administered for the last 24 h and the cells were collected for the radioactivity counting, as described in the Methods section. The results represent means ± SD from 5 independent measurements.

cells were undertaken. Analysis of the proliferation of NB-2a cells without or with the addition of 100 μM palmitoylcarnitine indicates that serum stimulated proliferation can be inhibited after more than 24 h, which corresponds to the length of a cell cycle of this cell line. This effect was observed to be even more pronounced after prolonged exposure to palmitoylcarnitine, reaching about 40 and 70% inhibition of cell proliferation after 48 and 72 h, respectively (Fig. 1). In further studies on the effect of palmitoylcarnitine, the thymidine incorporation was followed and, as presented in Fig. 2, the inhibitory effect of palmitoylcarnitine was concentration dependent. Since palmitoylcarnitine, being an amphiphile, can exert a slightly detergent-like effect on the plasma membrane (Lichtenberg et al. 1983), apart from its coupling to BSA, the conditions modifying the intracellular palmitoylcarnitine content were applied. As demonstrated by Nałęcz et al. (1995), administration of carnitine for even prolonged times resulted in uptake of this compound by NB-2a cells and the majority of carnitine was accumulated in the form of long-chain acyl derivatives. A more detailed analysis resulted in the detection of all the radioactivity of the pellet in a TLC spot corresponding to palmitoylcarnitine, with a R_f value higher than the long-chain derivatives of shorter acyl chain (Fig. 3). Carnitine at a physiological concentration for this compound (50 μM) (Bieber 1988) did not affect thymidine incorporation (Fig. 2), the addition of higher concentrations of carnitine inhibited proliferation of NB-2a cells by 30%.

Neuroblastomas have been described as highly undifferentiated tumors. Morphology of NB-2a cells, when this line has been cultured in EMEM with FBS, shows a majority of round cells with only few processes (Figs. 4A and 5A). Administration of either carnitine or palmitoylcarnitine was correlated with increased differentiation. The amount of processes increased significantly upon addition of either carnitine or palmitoylcarnitine (Fig. 4). There are also some vesicular structures visible in the reverse phase micrographs (Fig. 5). The formation of a complex network of processes is reminiscent of the morphology of the culture of cells isolated from the C 1300 Jackson tumor, where they were grown in the presence of horse serum and a rich nutrient mixture Augusti-Tocco and Sato 1969).

The activity of PKC was estimated, in order to decide if the observed effects of palmitoylcarnitine on proliferation and differentiation could result from the inhibitory effect on this kinase. For this purpose the modified method of Alexander et al. (1990) was applied, using a peptide substrate corresponding to the PKCα pseudosubstrate site, residues 19-31, with the exception that the alanine at position 25 is replaced by serine, resulting in a very high affinity (K_m = 0.2 μM) (House and Kemp 1987).

Phosphorylation of the peptide substrate increased with time (Fig. 6) and was stimulated about 2.5 times more by PDBu. This effect was stable from 8 to 24 min after phorbol ester administration, therefore a 12 min preincubation time with PDBu has been further applied. The activity of PKC was measured after the administration of various concentrations of palmitoylcarnitine. Titration with this long-chain carnitine derivative did not change the basic activity of PKC which turned out to be quite high for NB-2a cells (Fig. 7). The phosphorylation of peptide substrate stimulated by the addition of PDBu was, however, inhibited very strongly by palmitoylcarnitine in a concentration-dependent way, reaching 50% of maximal inhibition at 50 μM concentration.

Palmitoylcarnitine is a product of palmitoylcarnitine transferase I, catalysing an interconversion of long-chain fatty acyl-CoAs to fatty acylcarnitines. The amount of

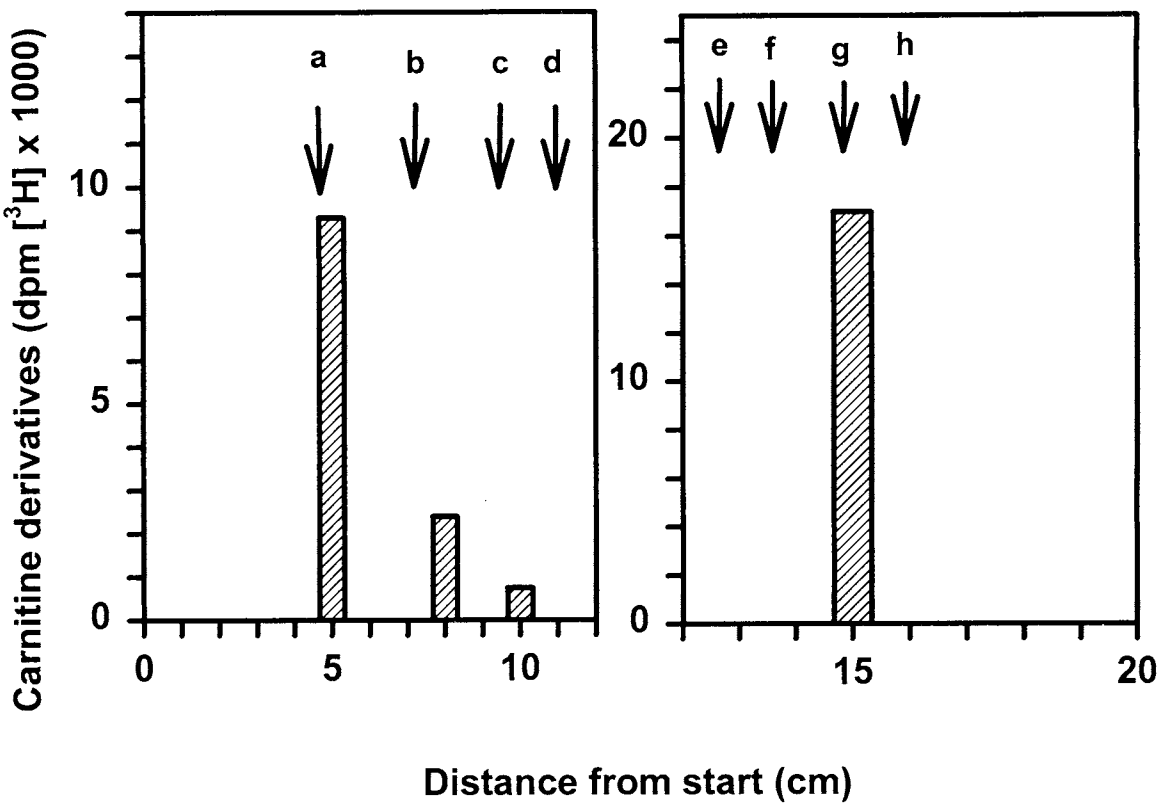

Fig. 3. Distribution of carnitine and its derivatives in neuroblastoma NB-2a cells. The cells were incubated for 2 h in the presence of 50 μM L-[methyl-^{3}H]carnitine (15 Ci/mol). The reaction was terminated by collecting the cells, TCA precipitation and further extractions were done, as described in the Methods section. TLC analysis was performed according to Huth et. al. (1981). The left panel presents the analysis of supernatant, the right one of the pellet obtained after TCA precipitation. The arrows marked a-h indicate the positions of carnitine and its acyl derivatives, namely free carnitine (a), acetylcarnitine (b), propionylcarnitine(c), hexanoylcarnitine (d), octanoylcarnitine (e), lauroylcarnitine (f), palmitoylcarnitine (g), stearoylcarnitine (h).

intracellular palmitoylcarnitine can be shifted by administration of carnitine. Supplementing the culture medium with increasing concentrations of carnitine resulted in an increased accumulation of free carnitine and its derivatives, the relative content of palmitoylcarnitine was, however, quite stable (57-68%) (Fig. 8A). The absolute content of palmitoylcarnitine increased from 350 pmoles/mg protein to 2.2 nmoles/mg protein when the carnitine concentration was raised from 50 to 500 μM (Table I). It increased even more dramatically with a further increase in carnitine concentration. Administration of etomoxir, a well established inhibitor of palmitoylcarnitine transferase I (Kiorpes et al. 1984) did not exert any effect on the content of palmitoylcarnitine when this inhibitor was added for shorter times than 24 h (not shown). Most probably its ability to penetrate the plasma membrane of neuroblastoma NB-2a cells is very low. The prolonged (48 h) incubation with etomoxir at 10 and 100 μM concentrations (comparable with those applied for brown adipocytes (Baht and Saggerson 1989)), followed by 2 h exposure to carnitine, resulted, as shown in Table I, in a significant decrease of palmitoylcarnitine content (by 30-42 %). Etomoxir did not affect the total accumulation of radioactive carnitine, the effect of this inhibitor changed the distribution of carnitine, with a dramatic increase in accumulation of free carnitine and the decrease of the relative content of palmitoylcarnitine (Fig. 8B). Unexpectedly, an effect on acetylcarnitine transferase was also detected, since the low content of acetylcarnitine was observed to decrease by 40% in the presence of 100 μM etomoxir (Fig. 8B).

The conditions known to either increase the intracellular content of palmitoylcarnitine or to decrease the absolute and relative amount of this long-chain acylcarnitine in NB-2a cells (Table I and Fig. 8) were applied in order to check the activity of PKC. As presented in Fig. 9, the basic phosphorylation of exogenous substrate measured without addition of phorbol ester did not

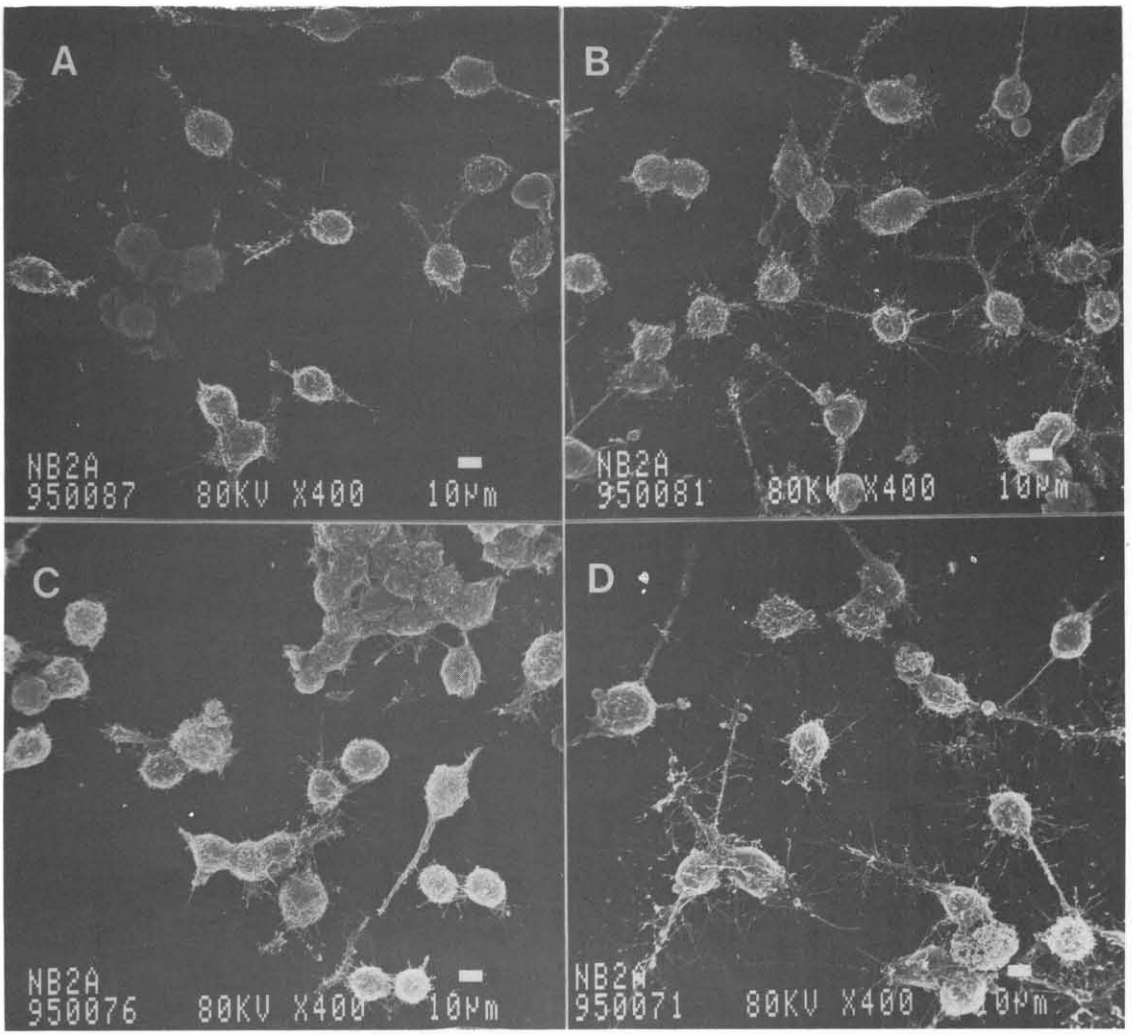

Fig. 4. Effect of carnitine and palmitoylcarnitine on differentiation of neuroblastoma NB-2a cells. Cells were seeded on polystyrol foil as with proliferation studies. After deprivation of serum for 24 h the incubation medium was supplemented for the next 48 h with 5% FBS, 95% EMEM either without any additions (A), or with 1 mM carnitine (B), BSA with ethanol (C) or 100 µM palmitoylcarnitine in ethanol, bound to BSA (D). Cells were fixed and prepared for scanning electron microscope as described in Methods. Bars represent 10 µm.

change upon addition of carnitine or exposure to etomoxir. The activity of PKC measured upon stimulation with PDBu was decreased by 28 and 40% after addition of 50 and 500 µM carnitine, respectively, for 2 h prior to the PKC activity assay (Fig. 9, bars A-C). A prolonged (48 h) exposure of neuroblastoma cells to 100 µM etomoxir, without addition of carnitine, did not affect the ability of PDBu to stimulate PKC activity to the same extent as in the control cells (Fig. 9, bars A and D). When, however, the cells treated with etomoxir were incubated for 2 h with 50 µM carnitine, its inhibitory effect on PKC activity was reversed (Fig. 9, bars E and F), practically reaching the control level, where the higher concentration of etomoxir was applied (Fig. 9, bar F).

DISCUSSION

The conditions under which the intracellular content of palmitoylcarnitine increased, i.e. administration of carnitine or incubation of the cells in the presence of palmitoylcarnitine, led to inhibition of cell proliferation with a parallel promotion of differentiation. The higher amount of processes after carnitine or palmitoylcarnitine treatment increased the content of vesicles within neurites - this can be interpreted as a stimulation of neurite outgrowth with a very active axonal transport (Hirokawa 1993). These observations would point to the fact that an elevated palmitoylcarnitine content in the cells promotes a cascade of events

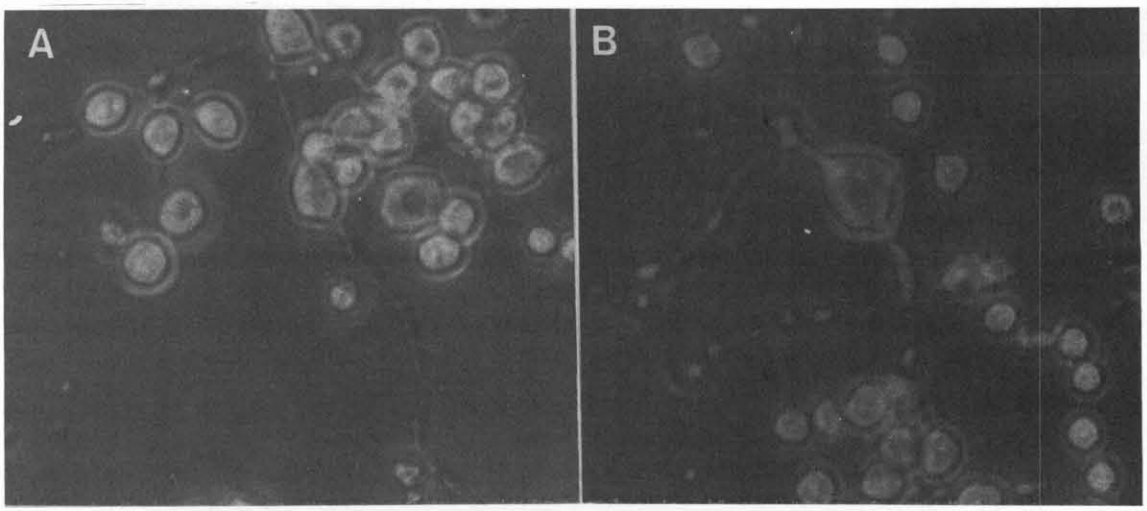

Fig. 5. Effect of carnitine on the formation of processes in neuroblastoma NB-2a cells. Cells were seeded on Petri dishes as for the proliferation studies and grown in the absence (A) or presence (B) of 1 mM carnitine. Phase microscopy performed with Nikon Phase contrast - 2 LWD 0.52, x 400.

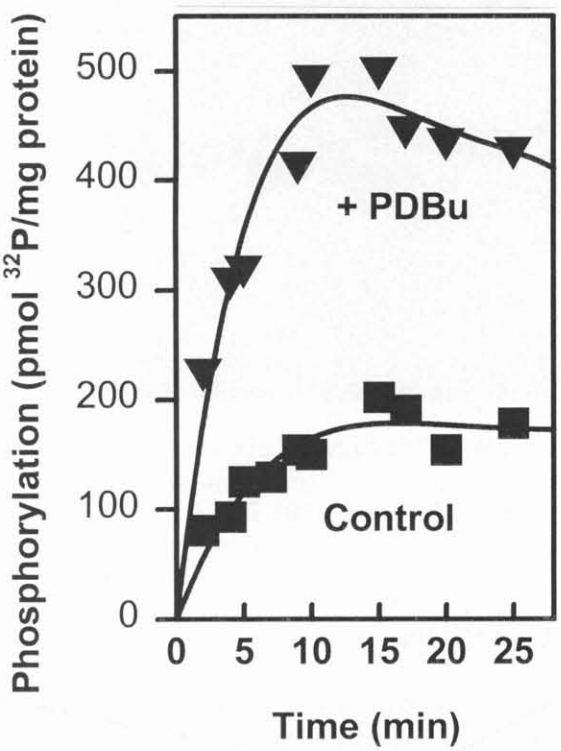

Fig. 6. Effect of phorbol ester on the activity of PKC in neuroblastoma NB-2a cells. Neuroblastoma NB-2a cells were collected by trypsinization and preincubated in the presence of SLO (6U/ml) for 25 min. PDBu (1 μM) was added at the indicated times related to administration of SLO. The reaction was started by the addition of peptide substrate and ATP and terminated after a subsequent 12 min. The reaction was stopped and the peptide was separated from ATP according to Alexander et al. (1990), with modifications indicated in the Methods section. The results represent the amount of ^{32}P incorporated into peptide referred to cellular protein.

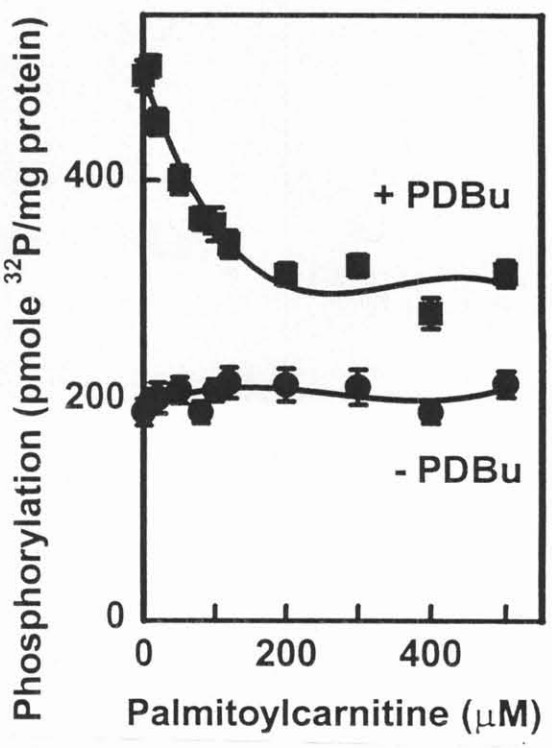

Fig. 7. Effect of palmitoylcarnitine on the activity of protein kinase C in neuroblastoma NB-2a cells. Cells were suspended in ICB and preincubated for 25 min in the presence of SLO (6U/ml), either without (circles) or with 1 μM PDBu (squares). Palmitoylcarnitine, at indicated concentrations, was given together with SLO and PDBu 12 min before the beginnning of the reaction. The reaction was started and terminated as given in the legend to Fig. 6 and in the Methods section. The results (means ± SD from 3 experiments) represent the amount of ^{32}P incorporated into peptide substrate referred to cellular protein.

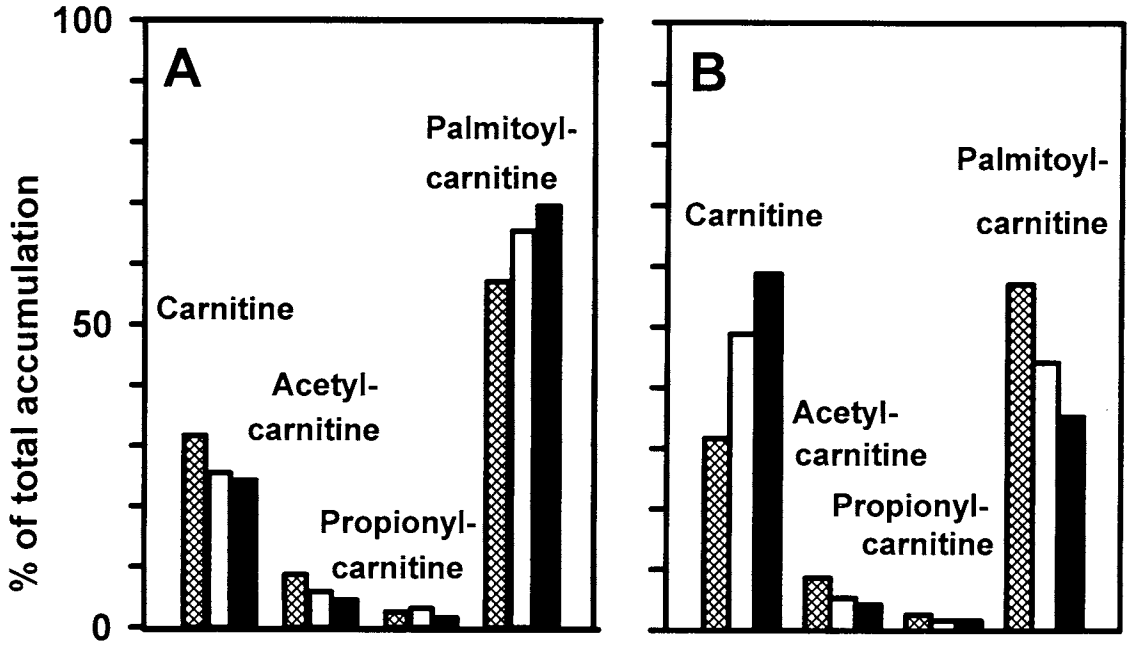

Fig. 8. Distribution of carnitine and its derivatives in neuroblastoma NB-2a cells. Cells were grown under standard conditions without any additions (A) or with etomoxir (B) for 48 h. [^{3}H]Carnitine at various concentrations (A) or at 50 μM (B) was administered for 2 h and cells were collected and long-chain acylcarnitines separated by TCA precipitation. The amount of free carnitine and its acyl derivatives were estimated as given in the Methods section. (A) Effect of various carnitine concentrations on the distribution of carnitine derivatives: Cells were incubated with either 50 μM (hatched bars), or 500 μM (open bars), or 1 mM (filled bars) carnitine. The results (means from 3 independent experiments) represent relative content of carnitine and its derivatives after incubation with the indicated concentration of carnitine. (B) Effect of etomoxir on the distribution of carnitine derivatives: Cells were preincubated either without any additions (hatched bars) or with 10 μM (open bars) or 100 μM (filled bars) etomoxir. The results (means from 3 independent experiments) represent the relative content of carnitine and its derivatives after preincubation with the indicated concentration of etomoxir.

TABLE I

Accumulation of palmitoylcarnitine in neuroblastoma NB-2a cells

Etomoxir (μM)	Carnitine (μM)	Palmitoylcarnitine accumulation (pmol/mg protein)
-	50	350 ± 45
-	500	2220 ± 175
-	1000	52950 ± 987
10	50	50242 ± 31
100	50	50204 ± 27

Cells were grown under standard conditions without any additions or with etomoxir for 48 h. Carnitine was administered for 2 h, the cells were collected and the amount of palmitoylcarnitine estimated, as described in the Methods section. The results represent means ± SD from 3 independent experiments.

switching the paths leading to proliferation into differentiation phenomena.

Due to earlier reports on the inhibitory effect of palmitoylcarnitine on the activity of PKC (Brockenbrough and Korc 1987, Nakadate and Blumberg 1987, Oh et al. 1988, Vescovi et al. 1988), the activity of this kinase was assayed *in vivo* upon modulation of the intracellular palmitoylcarnitine content. The increase of the amount of this long-chain carnitine derivative, observed after administration of different concentrations of carnitine, was not, however, proportional to carnitine concentration. This phenomen could be due to a complex system of carnitine accumulation in NB-2a cells (Nałęcz et al. 1995). This compound was described to be transported mainly by a carrier-dependent system at low concentration and by a facilitated diffusion at high concentration (Nałęcz et al. 1995). A lower accumulation of palmitoylcarnitine was detected after preatreatment of NB-2a cells with etomoxir. Although carnitine palmitoyltransferase was reported to be inhibited by 50 nM etomoxiryl-CoA (Kerner et al. 1994), we could not detect any effect of this com-

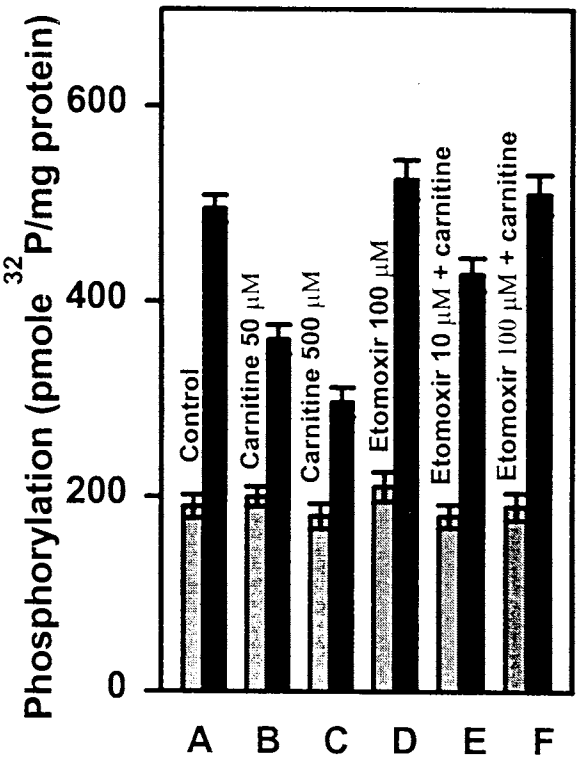

Fig. 9. Activity of protein kinase C in neuroblastoma NB-2a cells after modification of their intracellular palmitoylcarnitine content. Cells were grown under standard conditions either without any additions (A-C), or with etomoxir for 48 h (D-F). Carnitine at various (given) concentrations (B, C) or 50 μM (E, F) was administered for 2 h. Cells were collected, suspended in ICB and preincubated for 25 min in the presence of SLO (6U/ml) in the absence (grey bars) or presence (filled bars) of 1 μM PDBU (12 min). The reaction was started by the addition of peptide substrate and ATP and terminated as described in the Methods section. The results (means ± SD from 3 independent experiments) represent the amount of ^{32}P incorporated into peptide substrate referred to cellular protein.

pound on enzyme activity, therefore the time of exposure to etomoxir and the concentration were increased. Very little is known about the mechanism of etomoxir transport through the plasma membrane. Most probably is different in different cell types since the concentrations applied either to isolated or cultured cells varied from 1 μM (pancreatic islets of Langerhans (Zhou and Grill 1995)), to 50 μM (myocytes (Abdel-aleem et al. 1995)) and 300 μM (adipocytes (Baht and Saggerson 1989)).

The peptide used in the assay of PKC activity corresponded to the modified pseudosubstrate domain of PKCα. This peptide was demonstrated to be solely phosphorylated by PKC (House et al. 1987, Alexander et al. 1990). Moreover, the crystallographic studies confirmed that when compared with other protein kinases, the non-conserved amino acid residues were found at the surface responsible for the pseudosubstrate domain binding by PKC (Orr and Newton 1994). Due to the high degree of homology of the pseudosubstrate domain, the use of this peptide allows the measurement of phosphorylation activity of PKC in these cells. Otherwise it is difficult to estimate phosphorylation catalysed by PKC as a fraction of the total incorporation of [^{32}P] detected in the cellular pellet, what is pretty high (8.4 ± 0.6 nmol/mg protein). Only the atypical PKC isotypes, namely ε', λ and ς Burns and Bell Diaz-Meco et al. possess different pseudosubstrate domains, but their activity is not activated by phorbol esters (Newton 1995). It has to be emphasized that the substitution of alanine in position 25 for serine resulted in an excellent substrate for phosphorylation studies. Presence of several positive charges makes this peptide very easy to be separated from radioactive ATP in acidic millieu by binding to the negatively charged filters.

Under all the conditions studied so far, if either palmitoylcarnitine was added to permeabilized cells, or the intracellular level of this compound was risen by administration of carnitine, the activity of PKC stimulated by phorbol ester was decreased. Moreover, the inhibitory effect of palmitoylcarnitine was more pronounced with the raise of intracellular palmitoylcarnitine content. This effect was different to the observations reported on melanoma cells, in which case the incorporation of [^{32}P] was lower than in control cells when palmitoylcarnitine was below 88 μM (Vescovi et al. 1988). The stimulation of phosphorylation was, however, reported above this concentration. The activities measured at these very high (88 μmol/ml) palmitoylcarnitine concentrations (Vescovi et al. 1988) seem to be very difficult to be interpreted, since at the millimolar concentration range palmitoylcarnitine can act as a detergent. The, reported here, concentration dependent inhibition of PKC would point to the fact that the effect of palmitoylcarnitine can be limited to PKC isoforms which can be affected by phorbols (Burns and Bell 1992, Newton 1995).

It has been generally accepted that phorbol ester binding sites overlap with the site that can be occupied by diacylglycerol (Newton 1995). In our studies palmitoylcarnitine inhibited the phorbol ester stimulated reaction of phosphorylation catalysed by PKC. The cysteine-rich regions in the conserved C_1 domain of PKC isoforms are flanked with two positive and one negative charges (Bell

and Burns 1991), and this particular part of PKC molecule was proposed to be responsible for the binding of phorbol esters or diacylglycerol (Sharkey et al. 1984). It should be pointed out that the molecule of palmitoylcarnitine which posseses one positive and one negative charge and the increased density of electrons at the esterified carboxyl group, would be a perfect candidate for interaction with such a binding site.

If palmitoylcarnitine interacts with the phorbol/diacylglycerol binding site, one would expect some changes in the cytosol/membrane localization of PKC. In such a case either an inhibited transfer to the membrane and inhibited proteolysis to the active form of PKC or a faster down regulation and the proteolytic degradation of PKC would be observed. Both phenomena would result in lowering of PKC activity. An attempt to answer these questions has become the subject of further studies.

The observed and presented correlation of the increased palmitoylcarnitine content with lowering of the PKC activity in NB-2a cells would point to this long--chain carnitine derivative as another possible modulator of the activity of classical PKC types, especially as palmitoylcarnitine, unlike phorbol esters, occurs naturally in cells.

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ABBREVIATIONS

BSA	-	bovine serum albumin
EMEM	-	minimum essential medium with Earles salts
FBS	-	foetal bovine serum
PBS	-	phosphate bufferred saline
PDBu	-	12, 13- phorbol dibutyrate
PKC	-	protein kinase C
SDS	-	sodium dodecyl sulfate
SLO	-	streptolysin O
TCA	-	trichloroacetic acid

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