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INDUCTION OF ORNITHINE DECARBOXYLASE IN NORMAL AND PROTEIN KINASE C — DEPLETED HUMAN COLON CARCINOMA CELLS

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To examine the role of protein kinase C (PKC) in induction of human colon adenocarcinoma cell line, DETA/W, by polypeptide growth-promoting factors, ornithine decarboxylase activity (ODC) and DNA synthesis were determined in cells depleted of PKC. PKC depletion was achieved by prolonged cultivation (more than 30 passages) with 10^{-6} M phorbol 12-myristate 13-acelate. Lack of PKC in studied cells was proved by measurements of PKC activity and immunoreactivity. Although ODC activities and DNA syntheses in PKC-depleted cells were decreased by about 40—50% compared to normal DETA/W cells, the percentage increase of these mitogen-responsive reactions was quantitatively similar in both cell sublines. These results raise the possibility that not all of the biological responses to growth factors are connected with the activation of calcium-dependent PKC.

Key words: *protein kinase C, adenocarcinoma cells, ornithine decarboxylase, DNA synthesis*

INTRODUCTION

Ornithine decarboxylase (ODC) is the first, rate limiting, enzyme in the biosynthesis of polyamines (1, 2). Cell growth, regulated by numerous extracellular growth factors and hormones, is always accompanied by increased activity of ODC. However, although several distinct intracellular pathways are involved in response to growth factors or other external signals, it is still uncertain how external growth signals are transmitted from the surface receptors to the cell nucleus. In growth signal transduction pathways, activated membrane receptors initiate the cascade of events leading to

activation of several kinases. One of these kinases involved in activation of polyamine synthesis is protein kinase C (3—8).

The aim of this study was to evaluate the role of PKC in induction of proliferation of human colon carcinoma cell line by growth factors.

MATERIALS AND METHODS

Cells.

The human colon carcinoma cell subline, DETA/W, cultivated over 110 passages in Warsaw Cancer Center, was used for the experiments. The primary line DETA was kindly provided by Dr. M. Vetterlein, The Cancer Research Institute, Vienna.

Cells were grown at 37°C in complete RPMI 1640 medium supplemented with 10% fetal calf serum (Gibco), 2 mM glutamine, penicillin (100 units/ml), streptomycin (0.01%) and humidified with a 5/95% CO_2/air gas mixture in ASSAB Incubator.

Protein kinase C depleted DETA/W cell line, (DETA/W/PKC-), was obtained after prolonged cultivation with 10^{-6} M phorbol 12-myristate 13-acetate (PMA) (more than 30 passages).

General protocol.

0.5×10^6 cells were plated in 35 mm dishes in RPMI 1640+10% FCS+2 mM glutamine+antibiotics. Cells were allowed to grow for 5—7 days. Then, to provide a consistent starting point for the experiments, 18-hours cultivation in serum-free medium preceded the start of incubation with the test substances. At the end of the experiment, the dishes were placed on ice and rinsed twice with ice-cold PBS. After aspiration of the final wash, cells were scraped off and washed again with PBS. All experiments were performed in triplicate.

Ornithine decarboxylase assay.

PBS washed cells were resuspended in homogenizing buffer (0.25 mM pyridoxal-5-phosphate, 5 mM DTT, 1 mM EDTA, and 50 mM Hepes, pH=7.5). Cells were disrupted by sonication, and after centrifugation (12.000g, 4°C, 10 minutes) supernatants were stored at —80°C. ODC activity was measured by the method that quantitates the release of $^{14}CO_2$ from L-[1-^{14}C]ornithine (9). A 15 μl sample of the supernatant extract was mixed with 15 μl of reagent mixture (0.25 mM pyridoxal-5-phosphate, 5 mM DTT, 1 mM EDTA, 0.185 mM L-[1-^{14}C]ornithine hydrochloride (0.15 μCi/15 μl reagent mixture), and 50 mM Hepes, pH=7.5) inside a 1.5 ml Eppendorf tube capped with blue pipette tip. The pipette tip was fire sealed on its thinner end and it contained a Whatman 3M paper strip (2.5×1 cm) inside, which was wetted with 80 μl of Hyamine hydroxide. The tubes were incubated at 37°C for 60 minutes. Then, tubes were placed on ice, uncapped one at a time, and 15 μl of 2 M citric acid was added to each Eppendorf tube. The quickly recapped tubes were vigorously mixed and reincubated for another 60 minutes. At the end of the reincubation, paper strips were removed from the caps (pipette tips) and they were replaced in the counting vials containing liquid scintillation cocktail. Reaction blanks were prepared by using homogenizing buffer instead of cell extracts. All assays were done in triplicate.

The protein concentration was measured using Micro BCA Protein Assay, Pierce Chemical Co.

Deoxyribonucleic acid synthesis.

The rate of DNA synthesis was determined by adding 2 μCi of [^{3}H]thymidine per milliliter of culture medium. The radiolabelled precursor was added to cells after 18 hours of cultivation in serum-free medium. Following incubation with or without the test substances, cells were washed with ice cold PBS and scraped off. Then, one more wash with ice cold 0.2 M perchloric acid was done, cells were centrifuged (12.000 g, 4°C, 10 minutes), and pellets were kept in 0.3 M KOH for 60 minutes at 37°C with mixing. After centrifugation, supernatants were placed on ice for 10 minutes, centrifuged, and resulting pellets containing DNA were solubilized in 10% perchloric acid by heating to 70°C for 20 minutes. After final centrifugation, incorporation of ^{3}H-thymidine into DNA was determined by counting the radioactivity and the estimation of DNA concentration in the supernatant. The DNA content of the samples was measured by the fluorometric method (10). DNA synthetic activity was calculated as DPM per minute per microgram of DNA. All measurements were performed in triplicate.

Immunoreactive measurement of protein kinase C.

Cells washed in ice-cold PBS were resuspended in 2 ml of cold lysis buffer (20 mM Hepes, 2 mM $MgCl_2$, 10 mM EGTA, 2mM EDTA, 2 mM dithiothreitol, 0.5 mM PMSF, pH = 7.5) and homogenized with Dounce glass homogenizer at 4°C. The preparation was centrifuged at 100.000 x g for 60 min. at 4°C, and the supernatant (representing the cytosolic fraction) was saved for assay. The membrane pellet was resuspended in 1 ml of 0.1% Triton X-100 in lysis buffer, and after centrifugation (100.000xg) for 60 min. at 4°C was used for assay. The PKC immunoreactivity in cytosolic and membrane fractions was determined by electroblotting and immunostaining. Briefly, 20 μg of proteins mixed with loading buffer (60 mM Tris-HCl, pH=6.8, 2% SDS, 10%glycerol, and 5% ß-mercaptoethanol) was separated in 10% SDS-polyacrylamide gel. Proteins from the gel were transferred to PVDF membrane by use of MilliBlot-semidry transfer system. After excess of nonspecific binding sites on the membrane was blocked with 5% albumin, proteins were immunostained indirectly using a monoclonal antibody to protein kinase C (clone MC5 — Amersham), followed by anti-mouse Ig-peroxidase conjugate. Blots were developed in 0.05% 3,3'-Diaminozidine-4HCl, 0.03% H_2O_2 in PBS.

Measurement of protein kinase C activity.

PKC activity in cytoplasm and membranes was assayed by incorporation of ^{32}P into histone III-S as described previously (11).

Statistical analysis.

Results are presented as mean $\pm$ SD. Significant difference between the values was assessed by means of Student's test. A P value of < 0.05 was considered significant.

RESULTS

Treatment of cells with tumor-promoting phorbol esters, such as PMA, causes rapid translocation of the cytoplasmic PKC activity to the membrane, whereas prolonged PMA treatment results in down regulation of PKC (11—13).

As shown in *Table 1,* cultivation of human colon adenocarcinoma cell line — DETA/W cells for more than 30 passages with 10^{-6} M of PMA resulted in decrease ^{32}P incorporation into histone in the presence of phosphatidylserine, diolein, and Ca^{2+} to the values similar to those found in the presence of EGTA and absence of added phosphatidylserine, dolein, and Ca^{2+}, both in cytoplasmic and membrane fractions. Therefore, calcium-dependent PKC in DETA/W

Table 1. Protein kinase C activity (pmol P/min/μg) in cytosolic and membrane fractions of normal and PKC-depleted colon carcinoma DETA/W cells.

	Total kinase activity		Ca^{2+}, phospholipid independent kinase activity		Net PKC activity	
	Cytosol	Membrane	Cytosol	Membrane	Cytosol	Membrane
normal cells	2.05	1.74	0.30	0.38	1.75	1.36
PKC-depleted cells	0.34	0.45	0.38	0.47	—	—

Data are means for 3 different cell preparations.

cells grown in the presence of PMA for a long period of time was undetectable. In addition, as determined by Western blotting and immunostaining techniques, no protein kinase C immunoreactivity in both cytoplasmic and membrane fractions of PKC-depleted DETA/W cells was detected *(Fig. 1).*

Fig. 1. Cytosol (C) and membrane (M) protein kinase C immunoreactivity present in normal DETA/W and absent in PKC-depleted DETA/W cells.
Equal protein concentrations were made by appropriate sample dilution and mixing with loading buffer. Equal volumes of the samples were subjected to polyacrylamide gel electrophoresis, electrotransferred to PVDF membranes, and immunostained with monoclonal anti-protein kinase C. Approximately 80-kDa single band was found in the fractions extracted from normal but not from PKC-depleted cells.

Measurement of ODC activities in DETA/W cells, cultivated in serum-free medium, demonstrated that in normal cells the mean ($\pm$SD) ODC activity was 1.82 ± 0.07, and depletion of PKC produced the significant decrease in enzyme activity to a mean ($\pm$SD) of 0.97 ± 0.06 nmol $^{14}CO_2$/mg protein/hour. The data present results from 3 different cell preparations.

Eighteen-hour serum-deprived cells were treated with 10^{-6}M of human recombinant epidermal growth factor (EGF), 10^{-7}M of human recombinant interleukin-1α (IL-1), 5×10^{-8}M of gastrin or 10% fetal calf serum.

One-hour exposure of normal DETA/W cells to all mitogens produced significant increase of ODC activity over the basal levels, being the highest in response to EGF stimulation *(Fig.2)*. In DETA/W/PKC- cells enzyme activity significantly increased after one-hour incubation with EGF, gastrin or FCS.

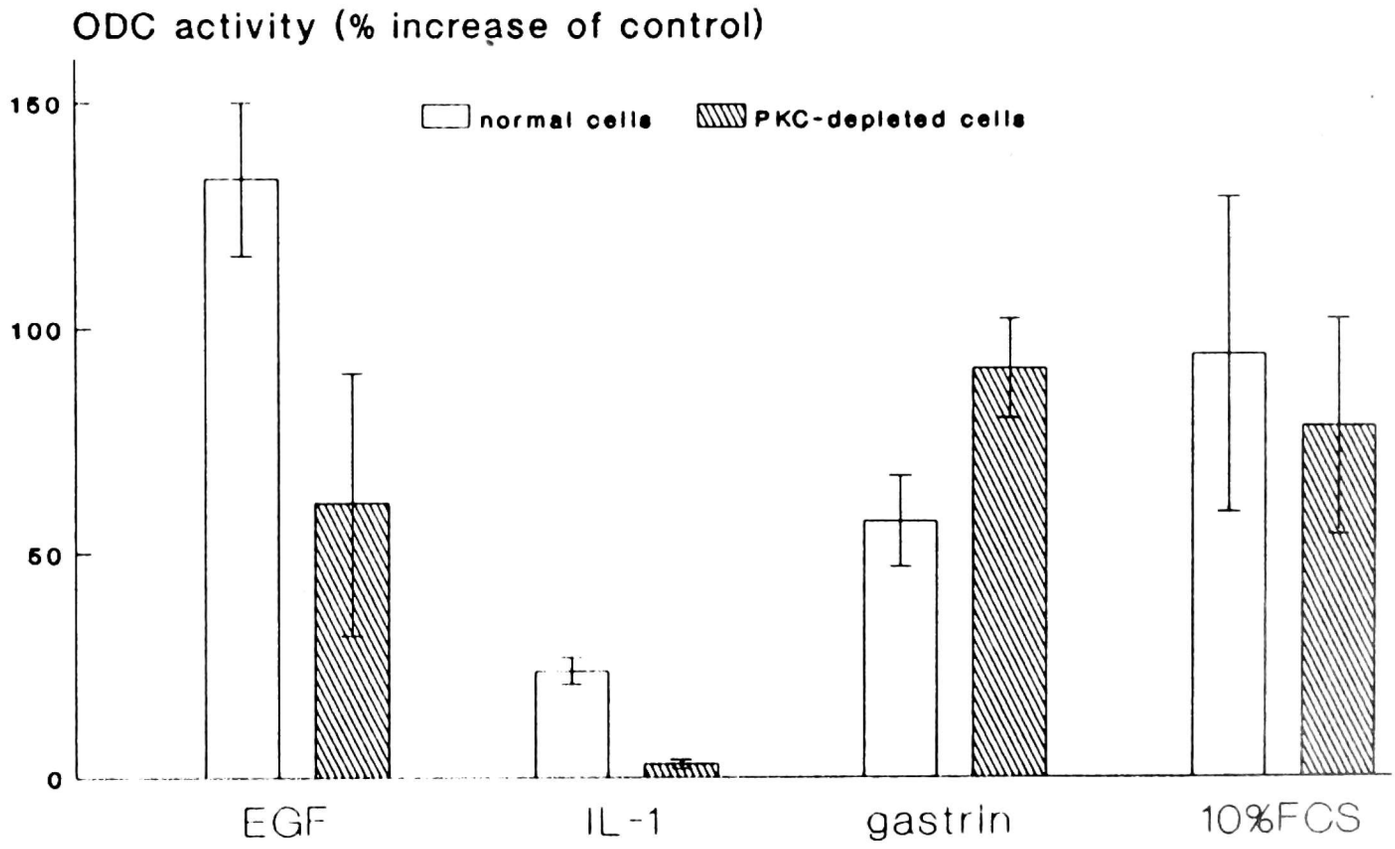

Fig. 2. Ornithine decarboxylase activity in normal and PKC-depleted DETA/W cells cultivated for one hour in the presence of polypeptide growth factors. Values are expressed as the percentage of increase relative to control value of unstimulated cells which was 1.82 ± 0.07 and 0.97 ± 0.06 nmol $^{14}CO_2$/mg protein/hour in normal and PKC-depleted cells, respectively. Data are means $\pm$ SD for 3 experiments.

Four-hour stimulation with mitogens produced significant increase of ODC activities to all test substance in both normal and DETA/W/PKC-cells *(Fig. 3)*. ODC activities increased about 14 times in response to serum and 6 times in response to EGF. Although ODC activities in PKC-depleted cells were decreased by 40—50% compared to normal DETA cells, the percentage increases in response to the mitogens used were very similar in the both sublines.

The DNA synthesis in DETA/W cells growing in standard conditions, as assessed by the incorporation of [^{3}H]thymidine into DNA, was significantly higher than that found in PKC-depleted DETA/W cells (45 ± 3 and 27 ± 4 DPM/min./μg DNA, respectively). Results represent 3 experiments performed on different cell preparations. As shown in *Figure 4*, the percentage increases in

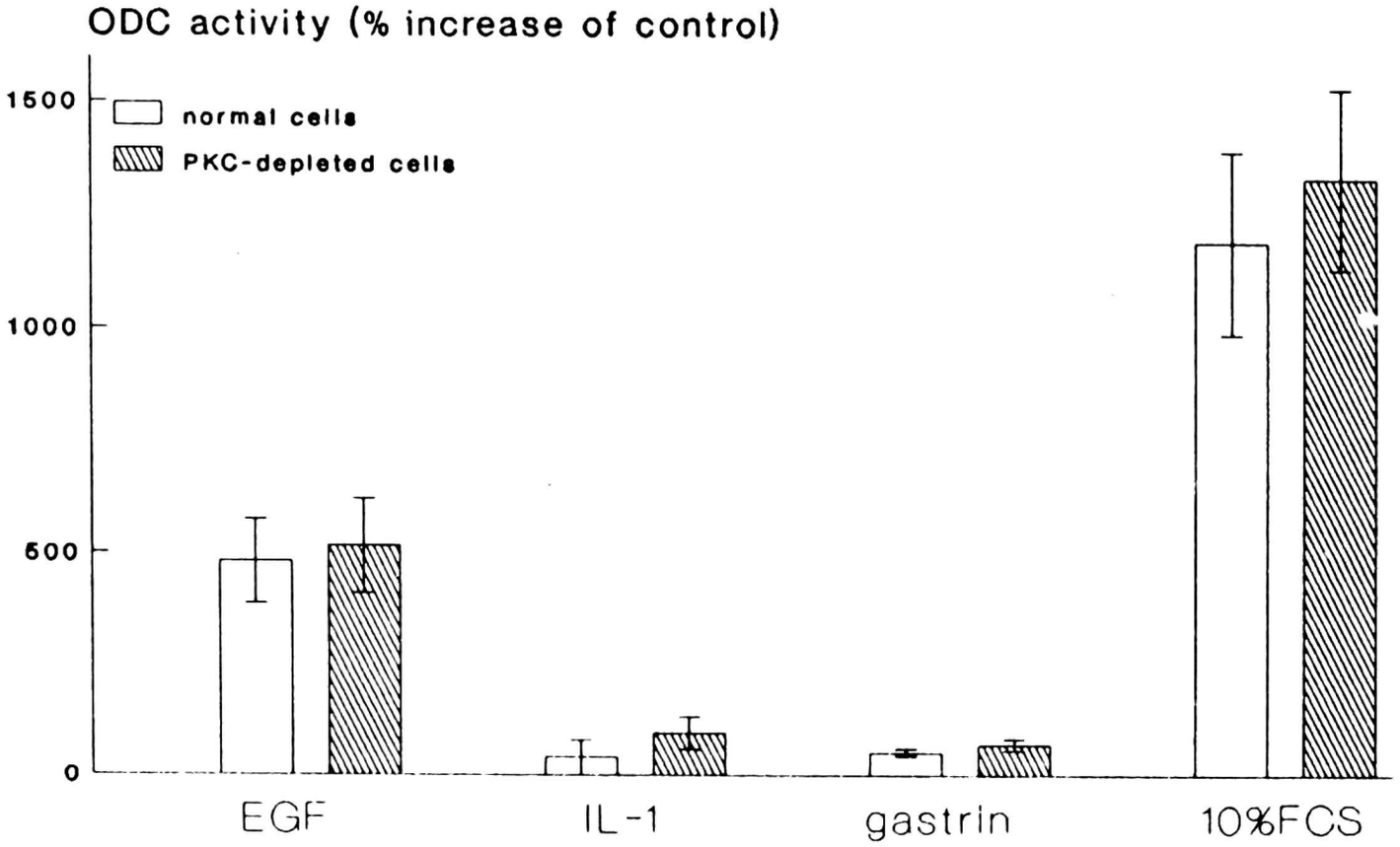

Fig. 3. Ornithine decarboxylase activity in normal and PKC-depleted DETA/W cells cultivated for four hours in the presence of polypeptide growth factors. Values are expressed as the percentage of increase relative to control value of unstimulated cells which was 1.82 ± 0.07 and 0.97 ± 0.06 nmol $^{14}CO_2$/mg protein/hour in normal and PKC-depleted cells, respectively. Data are means $\pm$ SD for 3 experiments.

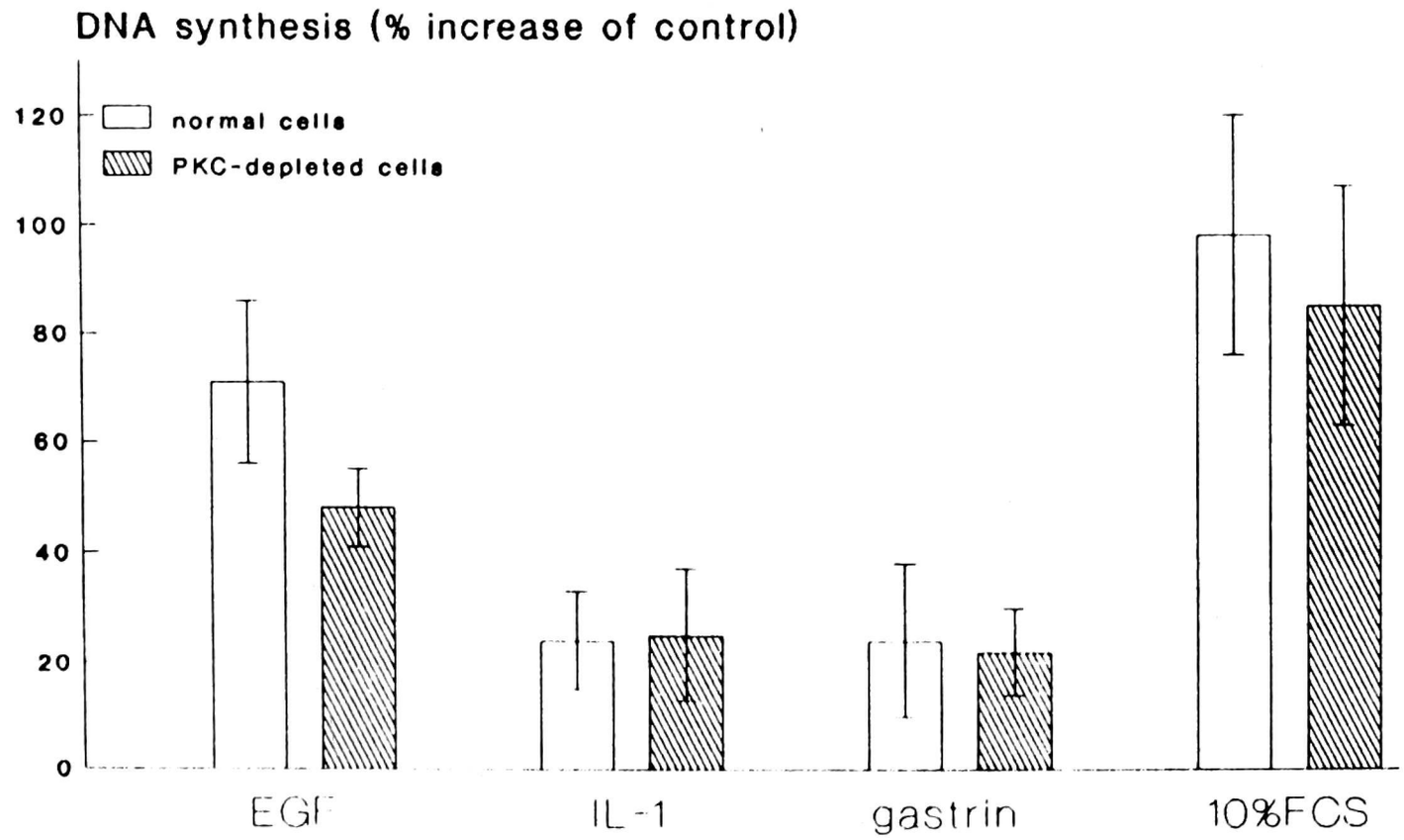

Fig. 4. [^{3}H]thymidine incorporation into DNA of normal and PKC-depleted DETA/W cells cultivated for four hours in the presence of polypeptide growth factors. Values are expressed as the percentage of increase relative to control value of unstimulated cells which was 45 ± 3 and 27 ± 4 disintegrations/min/μg DNA in normal and PKC-depleted cells, respectively. Data are means $\pm$ SD for 3 experiments.

thymidine incorporation over the basal levels of serum-deprived cells in response to four hour mitogen stimulation were quantitatively similar in normal and PKC-depleted sublines. The highest thymidine incorporations were stimulated by 10% FCS and these results corresponded to the highest ODC activities achieved in four hour response to serum in normal and PKC-depleted cells. Similarly to the lowest activation of ODC, the lowest DNA synthesis was found in response to IL-1 or gastrin treatment.

DNA synthesis assessed 24 hours following stimulation by growth factors showed also that percentage increases in thymidine incorporation were similar in normal and PKC-depleted cells, and the activation of thymidine incorporation after EGF and FCS treatment increased 6 and 10 folds, respectively, in both cell lines (data not shown).

DISCUSSION

This study has shown that polypeptide growth factors (EGF, gastrin, IL-1, and serum) still increase ODC activity in colon carcinoma DETA/W cells cultivated for months in the presence of 10^{-6}M PMA. Under described conditions, no calcium-dependent PKC activity or immunoreactivity could be detected in cytosolic or membrane fractions extracted from PKC-depleted cells. Our results are consistent with those published previously (12,13) on other cell lines that were made PKC — deficient by preincubation with higher PMA concentrations but for much shorter time.

Cell stimulation by growth factors leads to activation of a cascade of events involving several pathways. PKC activation is known to be associated with stimulation of several cell types proliferation, and PKC can cause an activation of numerous cellular events, including the rapid increases of transcription rate of certain genes. This latter phenomenon may be connected with the NF-*k*B release by PKC (14). The activation of PKC in response to growth-promoting factors results from the release of diacylglycerols from membrane inositol phospholipids and increase in cytosolic calcium ions stimulated by interactions of hormone with the membrane receptor.

The mechanisms which are involved in the rapid activation and turnover of ODC, an early event in cell replication, which is stimulated by growth factors, are still not enough elucidated. The activity of ODC is regulated at several levels, including enzyme synthesis and degradation, transcriptional step, postranslational modification, and interactions with macromolecules (4, 8, 12, 14—16). Besides numerous polypeptide growth factors, an activation of ODC can be obtained by PKC activators, such as phorbol esters, diacylglycerols, phospholipase C (3—8). On the other hand, inhibitors of PKC decrease phorbol ester induction of ODC (17—21), although the effectiveness of

staurosporine, one of a potent kinase inhibitor, may depend on the cellular and intracellular systems (22, 23). In addition, PKC activity can be inhibited by polyamines (24—27). Therefore, it is postulated that initial step in ODC induction by phorbol ester is connected with PKC activation. However, others suggest that although activation of PKC is involved in mechanisms of activation of ODC, this is not the sole event leading to ODC induction (28—29). Thus, the role of PKC in cell proliferation is still not clear.

The results of this study demonstrated that colon carcinoma DETA/W cells can grow even after they were completely depleted of calcium-dependent PKC activity. Moreover, the order of effectiveness of polypeptide growth factors for the activation of ODC and DNA synthesis was quantitatively similar in both normal and PKC-depleted cells, although these responses were inhibited in the absence of PKC by about 40—50%. Therefore, lack of PKC in DETA/W cells decreases but does not eliminate proliferative activity in response to growth-promoting factors. These results raise the possibility that, on the one hand, not all of the biological effects of growth factors are connected with the activation of PKC, and on the other hand, that the several cellular events activated by PKC during cell growth might be substituted by PKC-independent mechanisms connected with some other growth signal transduction pathways. While the above speculations are suggestive, the exact mechanism(s) of the described variability in the interactions between activation of PKC and cell growth remains to be elucidated.

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