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Protein Kinase C-Dependent and -Independent Pathways Mediate Epidermal Growth Factor (EGF) Effects in Human Endometrial Adenocarcinoma Cell Line KLE[1]

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The role of EGF in the proliferation of the poorly differentiated endometrial adenocarcinoma cell line (KLE) was examined. EGF (10 ng/ml) and the tumor promoter, protein kinase C agonist, phorbol 12-myristate 13-acetate (PMA) were potent stimulators ($P < 0.01$) of DNA synthesis in this cell line as determined by [^{3}H]thymidine incorporation into DNA. Staurosporine, a protein kinase C inhibitor, partially blocked the EGF effects on [^{3}H]thymidine incorporation. Similarly, downregulation of protein kinase C also failed to completely abolish EGF effect on DNA synthesis and cell division. These results suggest that in the KLE cell line EGF stimulation of cell growth is exerted through both protein kinase C-dependent and -independent pathways. © 1994 Academic Press, Inc.

INTRODUCTION

Endometrial cell growth is regulated by steroid hormones and growth factors[1,2]. Continuous stimulation of the endometrium by estrogen in the absence of progesterone is believed to set the stage for its malignant transformation [2]. Recent studies, however, have suggested that growth factors such as epidermal growth factor (EGF) may mediate the effects of estrogen in the endometrium[3].

The biological effects of EGF are mediated by the EGF receptor. The epidermal growth factor receptor (EGFR) belongs to a family of receptors with intrinsic tyrosine kinase activity [4] and has been implicated in the growth of malignant cells, probably through the protein kinase C-dependent pathway [5–7]. Following the binding of EGF by the receptor, a series of events such as an increase in ion flux and protooncogene expression is observed which ultimately results in increased DNA synthesis [3,8]. It has been reported that overexpression of EGFR in transfected NIH 3T3 cells produces a marked increase in DNA synthesis and confers a transformed phenotype in the presence of ligand [9]. Thus, EGF plays a mitogenic role in both normal and transformed cell types.

Previous studies from our laboratory have demonstrated the presence of EGF receptors [10] in human endometrial tissue. The specific binding was significantly different between normal and neoplastic endometrium, and an inverse relationship was observed between EGF receptor binding and tumor grade. We have also characterized the receptor in three endometrial cancer cell lines of varying grades and demonstrated a correlation between EGF receptor number and growth rate, implicating a role for EGF receptor in the growth regulation of these malignant cells [11]. In our continuing effort to understand the cellular mechanism of growth regulation of endometrial cells, in the present study we have examined whether protein kinase C may mediate the EGF effects on growth in a human endometrial carcinoma cell line KLE. The KLE cell line was chosen since it is derived from a highly undifferentiated type of endometrial adenocarcinoma.

MATERIALS AND METHODS

Mouse epidermal growth factor was purchased from Collaborative Research Inc. (Bedford, MA). Recombi-

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nant insulin-like growth factors I and II were a gift from Eli Lilly Research Laboratories (Indianapolis, IN). Cell culture media, EDTA, and bovine serum albumin (BSA) were obtained from Sigma Chemical Co. (St. Louis, MO). Disuccinimidyl suberate and BCA assay reagents were obtained from Pierce Chemical Co. (Rockford, IL), Chloramine T was from Kodak (Rochester, NY), and H stain (Hoechst 33258 dye) was from Aldrich (Milwaukee, WI). Electrophoresis reagents were obtained from Bio-Rad (Richmond, CA). Trypsin was purchased from Worthington Biochemical Corp. (Freehold, NJ).

Cell Culture

Human endometrial cancer cell line KLE (ATCC No. CRL-1622) was purchased from the American Type Culture Collection. KLE was maintained in DME/F12 mixture (Sigma D2906) supplemented with 10% fetal bovine serum, 50 μg/ml gentamycin, and 2 u/ml nystatin. Cells were cultured in 75-cm^2 tissue culture flasks (Corning Glass Works, Corning, NY) and maintained under sterile conditions at 37°C in water saturated air containing 5% CO_2. Subculturing was performed with (0.25%) trypsin/EDTA (0.02%) dissolved in phosphate-buffered saline (PBS).

[^{3}H]Thymidine Incorporation

[^{3}H]Thymidine incorporation assays were performed in 24-well plates [12]. Cells were plated at a density of approximately 200,000 cells/well for 24 hr in serum-free medium prior to any treatments. The plating efficiency under these conditions was greater than 90%. Cultures were then incubated for an additional period of 48 hr with various treatments. Four hours prior to completion, 1 μCi [^{3}H]thymidine was added to each well. Cells were then washed with ice-cold PBS followed by the addition of 5% TCA to remove the acid-soluble thymidine pool. Cells were again washed once with cold PBS and washed cells, with incorporated radioactivity, were dissolved in 500 μl of 0.2 *M* NaOH. This was transferred to scintillation vials containing 10 ml liquid scintillation Biosafe II. After neutralization with 0.2 *N* HCl, the radioactivity was determined using a beta counter.

DNA Assay

Cells were passaged and seeded into 24-well plates and treated with 10 ng/ml EGF as described previously for the thymidine incorporation assay. The DNA assay was performed by the method of Labarca and Paigen [13]. Briefly, after 48 hr of treatment, the medium was removed and the wells were washed with PBS. The cells were then solubilized using 0.5 *N* NaOH and then 0.75 ml DNA assay buffer (0.5 *M* $NaPO_4$ containing 2 *M* NaCl, and bisbenzimidazole (32 μg/ml) was added. Bisbenzimidazole (H stain) was used to enhance fluorescence. Calf thymus DNA standards were prepared and subjected to similar treatments. Spectrophotometric analysis was performed in replicates of 12 and expressed as nanograms DNA ± SEM. Fluorescence was measured at 356 nm (excitation) and 458 nm (emission) wavelengths.

Protein Kinase C Assay

The KLE cells were grown in 100-mm dishes, in the presence of 10% FBS, to confluency and incubated in serum-free medium for 24 hr prior to treatment. The cells were lysed in 5 ml lysis buffer (20 m*M* Hepes, 10 m*M* EGTA, 2 m*M* EDTA, and 1 *M* DTT), homogenized, and centrifuged at 100,000*g* at 4°C for 1 hr. The supernatant was chromatographed on DEAE sephacel columns after being equilibrated with column buffer (20 m*M* Hepes, 2 m*M* EGTA, 1 *M* DTT). Protein kinase C was eluted with column buffer containing 0.1 *M* NaCl, and its activity was ascertained by determining the amount of ^{32}P transferred from [γ^{32}P]ATP onto histones in the presence of Ca^{2+}, phosphatidylserine, and diolein. The phospholipid-independent protein kinases were assayed by performing incubations in the absence of phosphatidylserine and diolein. The amount of ^{32}P transferred onto histones in the absence of these phospholipids was then subtracted from the total activity to determine the protein kinase C activity.

Protein Assay

Protein concentrations in the DEAE eluates were measured using BCA assay reagent (Pierce, Rockford, IL). BSA was used as the protein standard.

Statistical Analysis

Statistical significance was determined at $P < 0.05$. Comparison of means was performed using the Student *t* test.

RESULTS

The mitogenic potential of EGF was evaluated using [^{3}H]thymidine incorporation into DNA as an indicator of mitogenic activity. A dose–response curve following 48 hr of exposure to EGF (Fig. 1) showed a significant increase in [^{3}H]thymidine incorporation at all concentrations tested with an optimum increase observed at 10 ng/ml ($P < 0.01$). To assess whether the plating density of these cells affected the response to EGF stimulation, similar studies were performed using the maximal stimulatory dose of EGF (10 ng/ml) with varying plating densities. A similar response ($P < 0.01$) to EGF was noted at all tested concentrations among the different cell densities (Fig. 2a). For the remainder of the experiments the

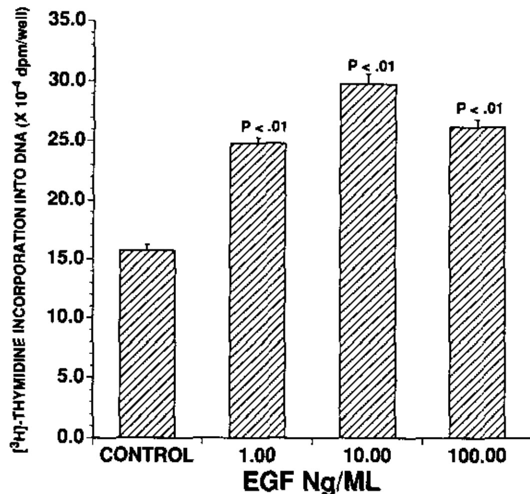

FIG. 1. Dose response of KLE cells to EGF on [^{3}H]thymidine incorporation. [^{3}H]Thymidine incorporation assays were performed in replicates of 12. Cells were plated at a density of 2×10^5 cells and treated with varying doses of EGF ranging from 0 to 100 ng. After 48 hr, cells were processed as described in the text.

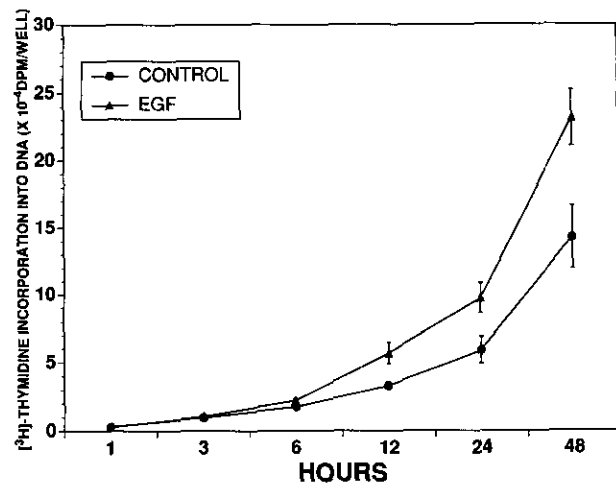

FIG. 2b. Time course of EGF on [^{3}H]thymidine incorporation. Cells were incubated in the presence or absence of EGF (10 ng/ml) for different time periods. The [^{3}H]thymidine incorporation assays were performed as described in the legend to Fig. 1.

cells were plated at high densities (2×10^5 cells/ml), and a time course (Fig. 2b) showed a progressive increase in [^{3}H]thymidine incorporation in EGF-treated cells compared to untreated cells at each interval from 6 to 48 hr of exposure ($P < 0.01$).

In order to correlate the increase in [^{3}H]thymidine incorporation with cell division, a quantitative analysis of cell number was performed in parallel with [^{3}H]thymidine incorporation assay. EGF exposure resulted in a near threefold increase in cell number compared to control

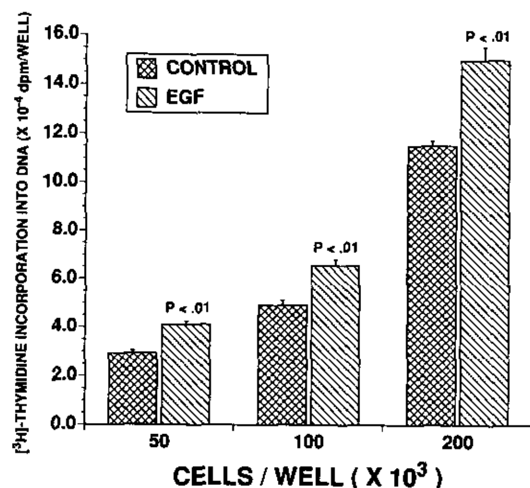

FIG. 2a. Effect of EGF (10 ng/mg) on [^{3}H]thymidine incorporation in cells cultured at different densities. [^{3}H]Thymidine incorporation assays were performed as described in the legend to Fig. 1. Cells were plated at varying densities ranging from 50 to 200 $\times$ 10^3 cells with or without EGF for 48 hr.

(28.31 ± 0.96 vs $84.43 \pm 0.82 \times 10^4$ cells/well). Similarly, DNA content increased significantly ($P < 0.0001$) in response to treatment with EGF (192.92 ± 6.67 vs 480.58 ± 8.87 ng DNA/well for control and EGF-treated cells, respectively). This further substantiates that an increase in [^{3}H]thymidine incorporation is representative of an increase in DNA synthesis and cell number.

After establishing the stimulatory effect of EGF on [^{3}H]thymidine incorporation into DNA, the mechanism by which EGF exerts this intracellular effect was investigated using modulators of protein kinase C. The tumor-promoting phorbol ester, PMA, and the inhibitor of protein kinase C, staurosporine, were used as tools to assess the role of protein kinase C activation in the growth enhancement of the KLE cell line. A dose–response curve of the effects of PMA on [^{3}H]thymidine incorporation into DNA showed that PMA was stimulatory ($P < 0.05$) at concentrations of 10^{-10} and 10^{-11} *M*, but inhibitory ($P < 0.01$) at 10^{-6} through 10^{-9} *M* (data not shown). A protein kinase C assay performed with these dosages confirmed that PMA at 10^{-11} *M* increased protein kinase C activity up to 100% (data not shown). Staurosporine, an inhibitor of protein kinase C, was then tested in the presence and absence of EGF. The dose–response curve for staurosporine alone (Fig. 3a) shows inhibition of [^{3}H]thymidine incorporation ($P < 0.01$) at concentrations above 10^{-9} *M* but not at 10^{-11} *M*. Studies using trypan blue stain concluded that the viability of these cells was not affected by staurosporine (data not shown). The effect of staurosporine on the responsiveness of KLE cells to EGF and PMA was then determined. PMA stimulation was inhibited by staurosporine (Fig. 3b), while the mitogenic response to EGF persisted in the presence of

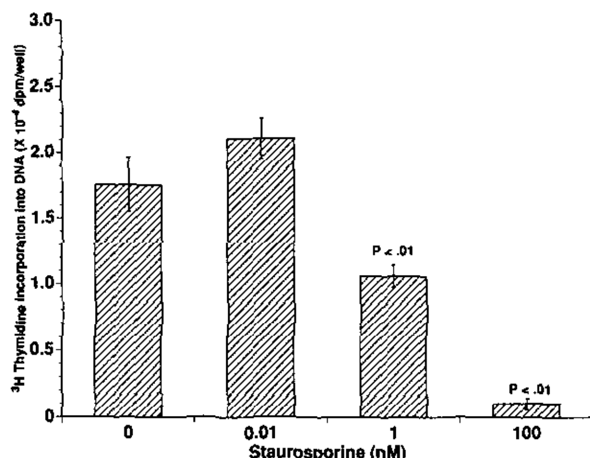

FIG. 3a. Dose-dependent effect of staurosporine on [^{3}H]thymidine incorporation. The assay conditions were similar to those described in the legend to Fig. 1.

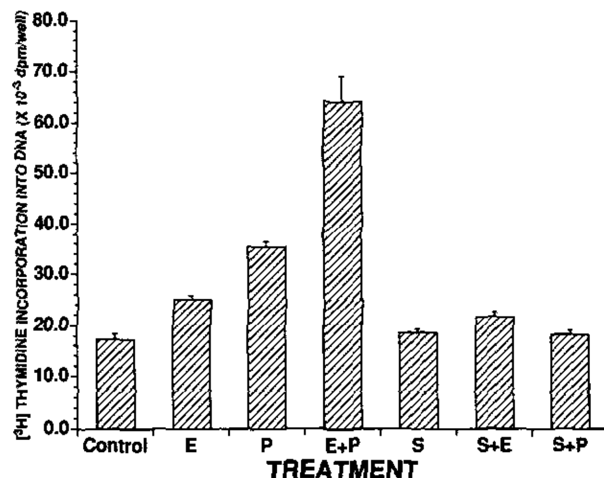

FIG. 3b. Effect of EGF (E) 10 ng/ml), PMA (P) 10^{-11} M, and staurosporine (S) 10^{-11} M alone and in combinations on [^{3}H]thymidine incorporation. The [^{3}H]thymidine assay conditions are described in the legend to Fig. 1.

staurosporine ($P < 0.01$). At the doses tested, PMA, EGF, and EGF in the presence of staurosporine augmented the mitogenic response, while staurosporine inhibited the mitogenic effects of PMA. This suggests that the inhibition of protein kinase C by staurosporine did not abolish the mitogenic response elicited by EGF.

Since staurosporine is a nonspecific inhibitor of protein kinase C, experiments were performed to further confirm that the effects of EGF are independent of protein kinase C activation. PMA was used at 1 μM to downregulate protein kinase C. KLE cells were preincubated for 48 hr in the absence or presence of 1 μM PMA followed by EGF at varying times (Fig. 4). EGF treatment in the absence of PMA preincubation resulted in a fivefold increase in protein kinase C activity following 5 min of incubation. However, EGF failed to increase protein kinase C activity when the cells were pretreated for 48 hr with 1 μM PMA. The 48-hr preincubation period with 1 μM PMA was chosen because when the cells were preincubated instead for 24 hr, protein kinase C could be induced by EGF toward the end of the treatment period (data not shown). Experiments were then performed to determine whether downregulation of protein kinase C by PMA would inhibit EGF effects on [^{3}H]thymidine incorporation. As shown in Fig. 5, KLE cells were preincubated with or without PMA for 48 hr. Cells were then washed and reincubated in the absence or presence of EGF (10 ng/ml) for 24 hr. The results show that the effects of PMA on [^{3}H]thymidine incorporation were inhibited when the cells had been preincubated with PMA for 48 hr. However, although the [^{3}H]thymidine incorporation in response to EGF in cells preincubated with PMA was lower than that in cells incubated with EGF alone, the amount of incorporation still remained significantly ($P < 0.01$) higher than controls. These results indicate that EGF was still able to increase DNA synthesis in cells in which protein kinase C was downregulated, further suggesting that EGF may exert its effects through protein kinase C-dependent and C-independent pathways.

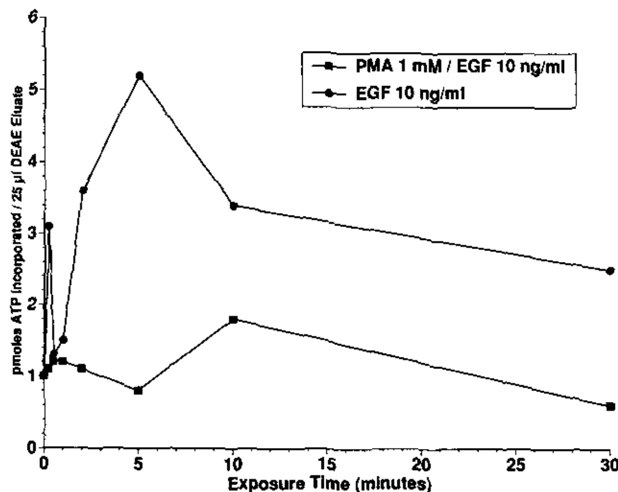

FIG. 4. PMA downregulation of PKC in KLE Cells. The cells were cultured for 48 hr in the absence or presence of PMA (1 μM). At the end of this incubation, the cells were washed and incubated with EGF 10 ng/ml for 0, 0.25, 0.5, 1, 2, 5, 10, and 30 min. At the end of each incubation, the cells were washed twice, lysed, homogenized, and centrifuged at 100,000g for 1 hr. The supernatant was chromatographed on DEAE sephacel columns and eluted with a 0.1 M NaCl buffer. Phosphotransferase activity was measured by the amount of [γ-^{32}P]ATP transferred to histones in the presence of Ca^{2+}, phosphatidylserine, and diolein. The data were expressed relative to the control.

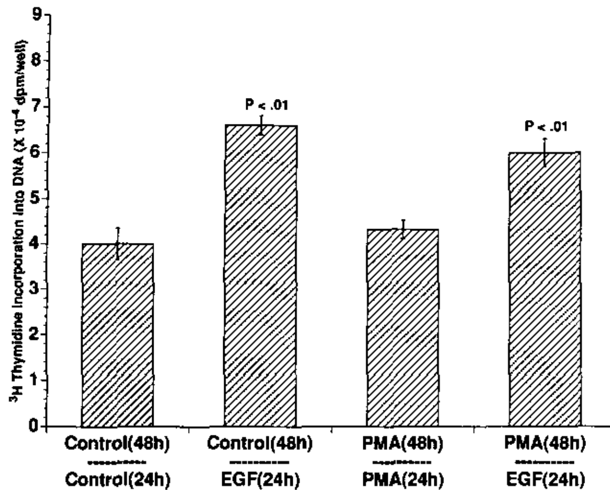

FIG. 5. Effects of protein kinase C downregutation by PMA on EGF stimulation. The cells were cultured for 48 hr in the presence or absence of PMA (1 μM). At the end of this incubation, the cells were washed three times and incubated in the presence or absence of alternate treatments for 24 hr. Following this, [*methyl*-[^{3}H]thymidine incorporation was measured.

DISCUSSION

The results from the present study show that the mitogenic activity of the KLE cell line, derived from a highly undifferentiated endometrial adenocarcinoma, may be regulated by EGF through interaction with its receptor. The increase in DNA synthesis observed with EGF stimulation was markedly enhanced when a protein kinase C agonist, PMA, was used as cotreatment. Although the increase in DNA synthesis in response to PMA was inhibited by staurosporine, a nonspecific inhibitor of protein kinase C (14), the effect of EGF was only partially blocked by the inhibitor. In addition, downregulation of the protein kinase C with 1 μM PMA did not completely abolish EGF effect on [^{3}H]thymidine incorporation. These results suggest that EGF may activate other mechanisms in addition to protein kinase C to increase DNA synthesis. In other studies using NIH 3T3 fibroblast cell lines, it has been shown that, although protein kinase C may be required for proliferation of these cells in response to fibroblast growth factor and platelet-derived growth factor (PDGF), activation of protein kinase C is not essential in order for the cells to respond to EGF [14].

The role of the EGF receptor in growth has been implicated by studies showing that a reduction in the extent of gene amplification and, consequently, in the number of EGF receptors expressed by A431 cells results in diminution of tumorgenicity in athymic mice [15]. There are various mechanisms through which EGF might regulate growth. One of the mechanisms is that of the activation of phospholipase C (PLC) $\gamma 1$, one of a family of isozymes that hydrolyzes PI 4,5-bisphosphate to produce IP_3 and diacylglycerol [16]. The former acts as a second messenger to liberate stored calcium from the endoplasmic reticulum and thereby activates calcium-requiring enzymes or processes, while the latter is an activator of protein kinase C. Another mechanism through which EGF may regulate growth involves the tyrosine kinase activation. The cytoplasmic tyrosine kinase domain of EGF receptor, as that of other growth factor receptor tyrosine kinases, is similar to a substantial number of oncogenes products possessing tyrosine kinase activity. Furthermore, the EGF receptor has a high degree of homology to the erbB-2 protein which is coded by the HER-2/neu oncogene [17]. Tyrosine kinase activity of the EGF receptor is likely to play a central role in the regulation of cell proliferation [18]. Our data support the notion that protein kinase C acts as a partial intermediary in the mitogenic activity of EGF. However, our data also suggest the involvement of a pathway independent of protein kinase C, since neither staurosporine nor downregulation of the protein kinase C pathway totally abolished EGF-stimulated responses.

In summary, the current study as well as the previous work from our laboratory [19] shows that EGF may play an important role in the growth regulation of the endometrial adenocarcinoma cell line KLE. While activation of protein kinase C leads to increased [^{3}H]thymidine incorporation into DNA, the present study suggests that EGF may act through pathways that are dependent and independent of protein kinase.

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