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EFFECTS OF GROWTH AND DIFFERENTIATION INDUCING FACTORS ON PROTEIN KINASE-C OF CULTURED INTESTINAL CRYPT CELLS

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SUMMARY: To assess the role of protein kinase-C (PK-C) in the growth and differentiation of small intestinal enterocytes, IEC-6 cells (a cell line derived from the crypts of rat small intestine) were incubated with factors known to induce growth (insulin, epidermal growth factor, gastrin, somatostatin and transferrin) or differentiation (transforming growth factor-β, retinoic acid and phorbol 12-myristate 13-acetate (PMA)). Cell proliferation (^{3}H-thymidine incorporation) and PK-C activity (Ca^{++}/phospholipid dependent) were measured. Among growth promoting factors only epidermal growth factor, insulin and transferrin were associated with increased ^{3}H-thymidine incorporation, and none of these agents induced PK-C activation as measured by its translocation from cytosol to membrane fraction. Of the differentiation inducing factors, only PMA translocated PK-C from cytosol to membrane. PMA also inhibited ^{3}H-thymidine incorporation in a dose dependent manner. These results suggest that growth and proliferation of enterocytes occur independent of PK-C signal transduction.

The small intestinal epithelial cell provides an interesting model to study the process of growth, differentiation and maturation. Mitotically active epithelial cells in the crypt region (Crypt cells) differentiate and mature as they migrate to the top of the villus to form non-dividing but functionally active villus TIP cells (1-3). Although a number of studies have examined the metabolism of small intestinal crypt and villus cells (4-6), the mechanisms of growth, differentiation and maturation of small intestinal crypt epithelial cells remains an important goal. Moreover, the epithelial cells provide a valuable model to elucidate the molecular mechanisms of growth and differentiation since they turn over every 72 hrs and

ABBREVIATIONS: PK-C, protein kinase-C; PMA, phorbol 12-myristate 13-acetate; EGF, epidermal growth factor; TGF-β, transforming growth factor-β; RA, retinoic acid

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several distinct enzyme markers of undifferentiated and differentiated epithelial cells are known.

Protein kinase-C (PK-C), a transmembrane signal in most proliferating cells, has been found in crypt cells, but not in TIP cells (7,8). The possible role of PK-C in the growth and differentiation of crypt cells has not been investigated. Quaroni and co-workers (9) have derived several cell lines from the rat small intestine which retain many of the antigenic and morphologic properties of crypt cells. In the present study, using a line derived from the crypts of the rat jejunum (IEC-6 cells), we have investigated the involvement of PK-C during proliferation in the presence of insulin, transferrin, epidermal growth factor (EGF), somatostatin, gastrin, and during differentiation in the presence of transforming growth factor-β (TGF-β), retinoic acid (RA) and phorbol 12-myristate 13-acetate (PMA).

MATERIALS AND METHODS

EGF and serum substitute ITS (insulin, transferrin, selenium and albumin) were purchased from Collaborative Research, Inc. Gastrin, somatostatin, transferrin, phorbol 12-myristate 13-acetate, phosphatidylserine, digitonin, 4α-phorbol 12,13, didecanoate, ATP and Dulbecco's Modified Eagle Medium (DMEM) were obtained from Sigma Chemicals (St. Louis, MO.). [γ-^{32}P] ATP 4000 Ci/mmol was purchased from ICN Radiochemicals (California). ^{3}H-thymidine was bought from New England Nuclear (Boston, MA.). TGF-β was generously provided by Dr. Anita Roberts, N.I.H.

IEC-6 rat small intestinal crypt epithelial cells were obtained from American Type Culture Collection (Rockville, MD). Cells were grown in 75 mm polystyrene flasks at 37°C in water saturated atmosphere with 5% CO_2 in air in either Dulbecco's Modified Eagle's Medium (DMEM) containing 5% heat inactivated fetal bovine serum (Flow Laboratories), 4 mM glutamine and 5 μg/ml insulin or serum free DMEM media containing ITS. The cells were harvested by trypsinization and reseeded in 25 mm diameter wells (tissue culture clusters from Costar, Cambridge, MA) at a density of 0.5 to 1.0x10^{6} cells/well. The cells were allowed to reach 50% confluency (24 hrs) before they were used for experiments.

The soluble and membrane bound PK-C fractions were isolated using the rapid procedure described by Pelech et al. (10). Briefly, the medium was removed and the cells were washed twice with 1 ml of cold PBS. The cells were incubated at 0°C for 5 min with 0.1 ml of buffer containing 20 mM Tris, pH 7.2, 2 mM EGTA, 2 mM EDTA and 0.5 mg/ml digitonin. The soluble extract was aspirated and the particulate extract obtained by solubilizing cell remnants in 0.2 ml above buffer containing 0.5% Triton X-100. Both soluble and particulate extracts were dialysed to remove digitonin and stored at -70°C until assay. The extracts were assayed directly or further purified on DE-52 ion exchange column as described previously (11).

Protein Kinase-C Assay: PK-C was assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into histone (12). The reaction mixture in a final volume of 100 μl contained 20 mM Tris-HCl (pH 7.4), 10 mM Magnesium acetate, 2 mM DTT, 250 μg/ml histone III-S., 80 μg/ml phosphatidylserine, 250 μM EGTA, 1 mM $CaCl_2$ and 5-10 μl cell extract containing 5-9 μg protein.

The reaction at 25°C was initiated by the addition of 5 nmole of [γ-^{32}P]ATP containing 0.5-1.0 x 10^6 CPM. After 10 min of incubation the reaction was stopped by adding 0.5 ml of ice cold trichloroacetic acid (TCA). In order to faciliate precipitation, few drops of bovine serum albumin (1 mg/ml) were added. The control tubes were run in an identical fashion except for the exclusion of Ca^{++}/phosphatidyl serine from the reaction mixture and these counts were substracted from the total counts to obtain specific protein kinase-C activity. The TCA precipitates were collected on Whatman GF/C filters, washed with cold 5% TCA and dried. Radioactivity was determined in a liquid scintillation counter using 5 ml of Fisher Scintiverse II (12).

Assay of DNA Synthesis: DNA synthesis was measured by the incorporation of ^{3}H-thymidine into the TCA precipitable fraction (13). Cells were grown to confluence (24 hrs) in 12 wells containing tissue culture clusters. They were then incubated in the presence or absence of the growth or differentiation inducing factors for the indicated period. Cells were pulsed with 1 µCi/ml of ^{3}H-thymidine during the last 6 hrs prior to harvest. The medium was then aspirated and the cells washed three times with ice cold PBS. Each well was then incubated at 4°C with 1 ml of 5% cold TCA for 30 min. The TCA was removed and the cells washed twice with cold TCA followed by cold 95% alcohol. The plates were air-dried and then incubated for 1 hr at 50°C in humidified air with 1 ml of 0.5 N NaOH. The resulting cell lysate was transferred to a scintillation vial, neutralized with HCl and radioactivity quantitated in a LKB liquid scintillation counter using 5 ml of Scintiverse II (Fisher Scientific).

RESULTS

The potential role of PK-C as a transmembrane signal during proliferation and differentiation of IEC-6 cells was investigated by examining ^{3}H-thymidine uptake and induction of PK-C activity in the presence and absence of selected growth and differentiation inducing factors.

The exposure of IEC-6 cells for 24 hrs to the growth promoting agents, EGF and a mixture of transferrin and insulin (10 µg/ml each) significantly enhanced ^{3}H-thymidine incorporation as compared to untreated control (Table 1). In contrast, ^{3}H-thymidine incorporation was unaffected by either gastrin or somatostatin.

The effect of these growth factors on PK-C activity in IEC-6 cells was next examined (Table 1). None of the growth factors induced PK-C activity in that most of the enzyme activity remained in cytosol fractions. A slight increase in particulate PK-C activity in the presence of EGF was not statistically significant.

Cells were subsequently treated with factors known to induce cellular differentiation (TGF-β (5), RA (14) and PMA (15,16)) and ^{3}H-thymidine uptake and PK-C activity were determined (Table 2). Neither TGF-β (10 nM) nor RA (10^{-8} M) affected either ^{3}H-thymidine uptake or PK-C activity. TGF-β at a higher

TABLE 1: Effect of Growth Factors on ^{3}H-Thymidine Incorporation and Translocation of Protein Kinase-C

Growth Factors	^{3}H-thymidine Incorporation CPM/10^6 cells	Protein Kinase-C Activity pmole/min/mg	
		Soluble Fraction	Particulate Fraction
Control	78,000 ± 2300	530 ± 72	173 ± 26
Insulin (5 µg/ml) Transferrin (10 µg/ml) Selenium (10 µg/ml) (ITS)	160,000 ± 8853	670 ± 30	195 ± 18
EGF (20 ng/ml)+ ITS	483,000 ± 9850	584 ± 55	206 ± 23
Gastrin (100 ng/ml)+ ITS	189,000 ± 9112	495 ± 65	250 ± 78
Somatostatin (100 ng/ml)+ ITS	166,000 ± 8475	710 ± 110	180 ± 60

Comparision of ^{3}H-thymidine incorporation and PK-C activity in IEC-6 cells exposed to growth inducing factors. Cells were allowed to reach 50% confluency. They were incubated in serum-free media containing ITS for 24 hrs for measurement of ^{3}H-thymidine incorporation or 30 min for assessment of PK-C activity in presence and absence of growth stimulating agents. The experimental conditions for ^{3}H-thymidine uptake and extraction and measurement of PK-C activity are described in Materials and Methods. Results are mean ± SEM of triplicates from three different experiments.

concentration (20 nM) slightly enhanced ^{3}H-thymidine incorporation (data not shown). In contrast, exposure to PMA (100 nM) resulted in 80% inhibition of ^{3}H-thymidine incorporation. Moreover, following incubation with PMA, a sig-

TABLE 2: Effect of differentiation factors on ^{3}H-thymidine incorporation and translocation of Protein Kinase-C

Differentiating Factors	^{3}H-Thymidine Incorporation CPM/10^6 cells	Protein Kinase-C Activity pmole/min/mg	
		Soluble Fraction	Particulate Fraction
Control	74,668 ± 5259	580 ± 60	215 ± 30
TGF-β (0.2 ng/ml)	62,297 ± 9659	620 ± 38	245 ± 69
Retinoic Acid (10^8M)	58,535 ± 8995	495 ± 83	287 ± 24
4β-Phorbol 12-Myristate 13-Acetate (100 nM)	23,400 ± 7652	216 ± 22	675 ± 45
4α-Phorbol 12,13 didecanoate (100 nM)	85,600 ± 6777	658 ± 73	198 ± 27

Comparison of ^{3}H-thymidine incorporation and PK-C activity in IEC-6 cells exposed to differentiation promoting factors. Subconfluent cells were incubated in serum-free media for 24 hrs for measurement of ^{3}H-thymidine incorporation or 30 min for measurement of PK-C activity. The experimental conditions are described in Materials and Methods. Results are mean ± SEM of triplicates from three different experiments.

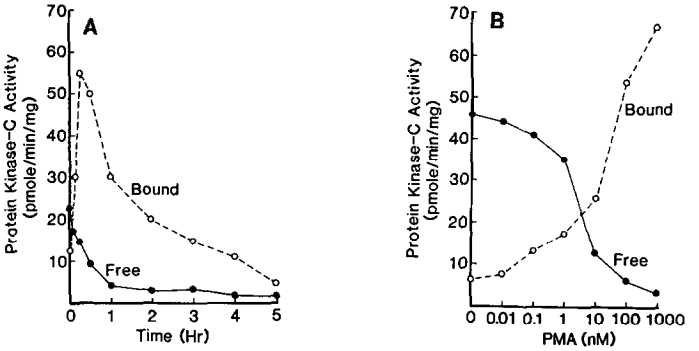

FIG. 1: Distribution of PK-C activity in cytosol and particulate fractions in PMA treated IEC-cells as a function of time (Panel A) and PMA concentration (Panel B). Subconfluent cultures of IEC-6 cells in plates (12 wells/plate) were incubated in serum-free media with 100 nM PMA for 5 to 240 min (A) or incubated for 30 min with 0 to 1 μM PMA (B). Free and membrane bound fractions were prepared and enzyme activity was assayed by ^{32}P incorporation into histone H-III. Each value is the mean ± SEM of determination from triplicate wells.

nificant portion of PK-C activity was translocated from cytosol to membrane fraction (Figure 1). As shown in Fig. 1(a), when cells were exposed to PMA for various lengths of time, particulate bound activity dramatically increased and peaked within 15 min., while cytosolic PK-C activity decreased. Moreover, when cells were treated with PMA for a longer time, the membrane bound PK-C activity was drastically reduced and fell below control levels after 4 hrs. The effect of PMA on translocation of PK-C was dose dependent and increased continuously between 10 ng/ml and 1000 ng/ml (Fig. 1(b)).

DISCUSSION

The effects of EGF, insulin, gastrin and lactoferrin on DNA synthesis and cellular proliferation associated with cell growth have been studied extensively in many cell lines (5,6,17,18). In the present report, we show that the proliferation and growth of small intestinal crypt cells can also be affected by EGF, insulin and transferrin. Despite the proliferative response induced by these agents, none of the factors were associated with activation of PK-C. Surprisingly, neither gastrin nor somatostatin stimulated proliferation of IEC-6 cells, as has been described in other reports.

TGF-β and RA had no effect on either ^{3}H-thymidine incorporation or PK-C activity. We cannot exclude the possibility that the lack of an observed

effect may be due to the absence, or a decreased number of TGF-β receptors on IEC-6 cells as a result of trypsinization of cells during passage(4).

One of the early events of PMA action in lymphocytes is the activation of PK-C and translocation of this enzyme to the membrane (20). Our data indicate that PMA renders a similar effect in IEC-6 cells, however, PMA decreases ^{3}H-thymidine incorporation at all concentrations. Moreover, biologically inactive phorbol ester 100 nm (4α-phorbol 12,13-didecanoate) failed to inhibit DNA synthesis or activate PK-C. Since a decrease of DNA synthesis is normally an early indication that cells are undergoing differentiation, it is not known whether PMA induces differentiation of IEC-6 cells in the present experiments.

Although the functional role of PK-C in cellular differentiation and proliferation is unknown, the induction of PK-C may be an essential process during signal transduction of physiological stimuli (19,20). PK-C is found in actively dividing crypt cells of the small intestine but not in villus TIP cells (7,8). The present study localizes PK-C to both the cytosol and membrane fractions of IEC-6 cells. Although EGF and insulin significantly stimulated the proliferation of IEC-6 cells, they had no effect on PK-C activity. We conclude that PK-C is not involved in the proliferation of intestinal epithelial cells.

Activation of PK-C by PMA results in decreased ^{3}H-thymidine incorporation. It is possible that PK-C may negatively control the proliferation of IEC-6 cells possibly through the down regulation of EGF and insulin receptors (21,22). It has recently been reported that the number of EGF receptors were significantly reduced in in vitro transformed IEC-18 cells as compared to non-transformed controls (4). Analagously, the number of insulin receptors on rat crypt epithelial cells decreases following partial small bowel resection (23) and this loss of insulin binding affinity coincides with decreased proliferative activity in crypt epithelial cells (23). Despite the opposing effects of PMA and EGF on the proliferation of IEC-6 cells, it is reasonable to hypothesize that the protein phosphorylation medi-

ated by PK-C may be be involved in Ca^{++} and Na^{+}/K^{+} transport mechanism. As a final comment, the potential role of PK-C in the differentiation of small intestinal crypt cells needs further investigation.

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