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Phorbol ester induces loss of VIP stimulation of adenylate cyclase and VIP-binding sites in HT29 cells

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Treatment of HT29 cells with the tumor promoting phorbol ester PMA resulted in an attenuation of VIP-stimulated cAMP production in intact cells and VIP-stimulated adenylate cyclase activity in cell membranes. PMA did not decrease the ability of cholera toxin and forskolin to elevate cAMP levels in intact cells. Fluoride-stimulated adenylate cyclase activity in HT29 cells homogenates was not affected by PMA. The maximal VIP binding capacity of homogenates prepared from HT29 cells treated with PMA was decreased by 50%. It is concluded that protein kinase C regulates VIP receptor function possibly through phosphorylation of the VIP receptor.

Phorbol ester; VIP; Adenylate cyclase; VIP receptor; Protein kinase C; (Human colonic HT29 cell)

1. INTRODUCTION

It is now well established that VIP exerts its physiological effects through binding to specific cell surface receptors and stimulation of adenylate cyclase [1]. In particular, the binding, molecular and functional properties of VIP receptors have been extensively studied in cellular and membrane systems derived from intestinal epithelium where VIP is thought to regulate water and electrolyte secretion [1]. A particularly useful model to study the molecular mechanisms of VIP action has been the HT29 cell line which is derived from a human colon adenocarcinoma [2]. This cell line has been shown to possess high-affinity VIP receptors which are very efficiently coupled to adenylate cyclase activation [3].

Here, we present evidence that the tumor-promoting agent PMA, a potent activator of protein kinase C [4], decreases VIP stimulation of adenylate cyclase and VIP-receptor binding in HT29 cells. It is proposed that protein kinase C modulates VIP action possibly through phosphorylation of the VIP receptor.

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Abbreviations: PMA, 4β-phorbol 12-myristate 13-acetate; 4α-PDD, 4α-phorbol 12,13-didecanoate; VIP, vasoactive intestinal peptide; N_s, stimulatory GTP-binding protein of adenylate cyclase; IBMX, isobutylmethylxanthine

2. MATERIALS AND METHODS

2.1. *Materials*

Porcine VIP was provided by Professor V. Mutt (Karolinska Institute, Stockholm). ^{125}I-labeled VIP (spec. act. 750 Ci/mmol) was prepared as described [5]. Forskolin, cholera toxin, IBMX, PMA and 4α-PDD were from Sigma.

2.2. *Cell culture*

HT29 cells were obtained from Dr J. Fogh (Sloan Kettering Institute for Cancer Research,

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Rye, NY). For measurements of cellular cAMP levels, cells were propagated in Falcon 75 cm^2 culture flasks and cultured for 3–5 days before use in Falcon 24-well plates as described [6]. For adenylate cyclase activity measurements and binding experiments, cell culture and cell particulate fraction preparation were performed as in [7].

2.3. *Cyclic AMP measurements*

Cyclic AMP levels in HT29 cells were measured by prelabeling the intracellular ATP pool with [^{3}H]adenine and purifying cellular [^{3}H]ATP and [^{3}H]cAMP exactly as in [8]. VIP and forskolin were exposed to HT29 cells for 10 min in 25 mM Hepes-Tris buffer, pH 7.4 [8]. Cholera toxin was incubated for 1 h with HT29 cells in their culture medium. All incubations were carried out at 25°C with 1 mM IBMX. Results were expressed as the percent of conversion of [^{3}H]ATP in [^{3}H]cAMP.

2.4. *Adenylate cyclase activity and ^{125}I-VIP-binding assays*

VIP-stimulated adenylate cyclase activity in HT29 crude particulate fractions was assayed as in [9]. ^{125}I-VIP-binding studies were performed as described in [7].

2.5. *Cell treatment with PMA*

In all experiments with the phorbol ester PMA, this agent at 100 ng/ml was incubated for 1 h at 37°C with HT29 cells in their culture medium. The cells were then used either for cAMP measurements or for the preparation of crude particulate fraction.

3. RESULTS

3.1. *Effect of PMA on VIP-stimulated cAMP production in HT29 cells*

As reported in [3], VIP exerted a potent and very efficient stimulation of cAMP production in HT29 cells (fig.1). Basal cAMP production was barely detectable with the technique used here to measure cAMP level. This made it difficult to estimate the maximal stimulation (over basal) induced by the peptide. Pretreatment of HT29 cells with PMA did not modify the EC_{50} value (2 nM) for VIP but resulted in a 40% decrease of VIP-induced cAMP accumulation at all peptide concentrations tested

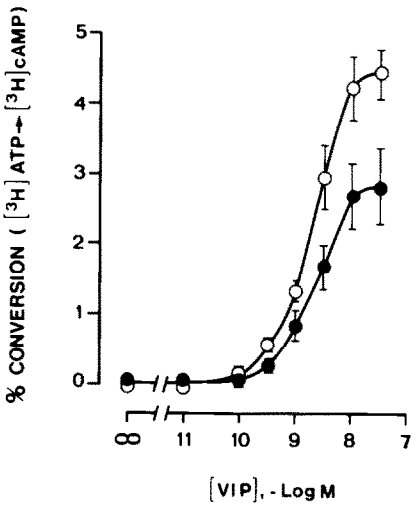

Fig.1. Concentration-response curves of VIP-stimulated cAMP production in control (○) or PMA-treated (●) HT29 cells. cAMP levels are expressed as the percent of conversion of [^{3}H]ATP in [^{3}H]cAMP. Data are means ± SE from triplicate determinations in a typical experiment. Similar results were obtained in another experiment.

(fig.1). In contrast, PMA did not decrease the ability of forskolin and cholera toxin to stimulate cAMP production in HT29 cells (table 1). The biologically inactive phorbol ester 4α-PDD had no significant effect on VIP-induced cAMP accumulation in HT29 cells (not shown).

3.2. *Effect of PMA on VIP stimulation of adenylate cyclase activity in HT29 cell membranes*

VIP markedly stimulated adenylate cyclase activity in a particulate fraction prepared from HT29 cells (fig.2). The EC_{50} for VIP was 0.16 nM. Pretreatment of HT29 cells with PMA decreased the peptide potency by about 2-fold (EC_{50} 0.3 nM) and also reduced by 20–30% the maximal extent of adenylate cyclase stimulation by VIP (fig.2). PMA was without significant effect on either basal (fig.2) or NaF-stimulated adenylate cyclase activity. Indeed, the enzyme activity stimulated by 10 mM NaF was 1340 pmol cAMP/min per mg protein in control membranes and 1370 pmol cAMP/min per mg protein in membranes prepared from cells pretreated with PMA.

Table 1

Effect of PMA on forskolin- and cholera toxin-stimulated cAMP production in HT29 cells

		Control	PMA
VIP	0.03 μM	100	63.6 ± 14.0
Forskolin	1 μM	13.1 ± 1.4	13.7 ± 1.6
	10 μM	70.7 ± 4.0	86.8 ± 12.1
	100 μM	258.6 ± 26.3	226.3 ± 16.2
Cholera toxin	0.01 μg/ml	11.4 ± 0.9	13.5 ± 0.9
	0.1 μg/ml	38.4 ± 1.9	41.7 ± 7.3
	1 μg/ml	157.8 ± 8.7	161.2 ± 7.7

Results are expressed as percent of cAMP production induced by a maximally effective concentration of VIP. Basal cAMP production in this series of experiments was less than 1% of the maximal stimulation by VIP. Each value is the mean ± SE from 3–4 determinations

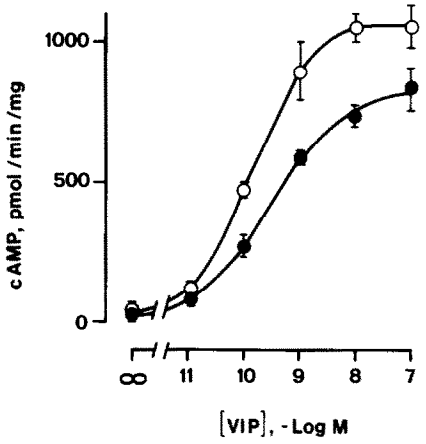

Fig.2. Concentration-response curves of VIP-stimulated adenylate cyclase activity in membranes prepared from control (○) or PMA-treated (●) HT29 cells. Enzyme activity is expressed in pmol/min per mg membrane protein. Data are means ± SE from two experiments with triplicate determinations in each experiment.

3.3. *Effect of PMA on VIP binding to HT29 cell membranes*

Competitive inhibition of ^{125}I-VIP binding by increasing concentrations of unlabeled VIP was performed in particulate fractions from control or PMA-treated HT29 cells (fig.3A). The phorbol ester decreased ^{125}I-VIP binding by about 50% at all unlabeled VIP concentrations. Scatchard

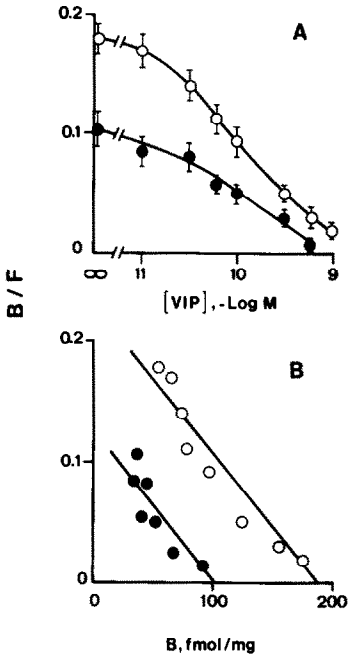

Fig.3. Competitive inhibition of ^{125}I-VIP binding by unlabeled VIP in homogenates of control (○) or PMA-treated (●) HT29 cells. In A, the ratio of bound over free (*B/F*) ligand concentration is plotted as a function of VIP concentration. Data are means ± SE from four experiments with duplicate determinations in each experiment. In B, the results are plotted according to Scatchard and were fitted by linear regression analysis.

analysis of data derived from 4 independent experiments (fig.3B) revealed that PMA did not modify the affinity of VIP for its receptors (K_d values of 0.17 ± 0.09 and 0.16 ± 0.09 nM in membranes from control and PMA-treated cells, respectively) but decreased maximal binding capacity by about 50% (B_{max} values of 200 ± 45 and 103 ± 22 fmol/mg protein in membranes from control and PMA-treated cells, respectively). It was checked that when added to a particulate fraction prepared from control HT29 cells, PMA did not interfere with ^{125}I-VIP binding (not shown).

4. DISCUSSION

The present data clearly show that treatment of HT29 cells with the phorbol ester PMA impairs VIP-induced increase in cellular cAMP levels and VIP-stimulated adenylate cyclase activity in cell membranes. It is now well established that the

stimulation of adenylate cyclase by VIP in HT29 cells and other cell types involves three protein components in the plasma membrane: the VIP receptor, the catalytic unit of adenylate cyclase and the GTP-binding protein N_s which couples the VIP receptor to the catalytic unit [1]. Thus, impairment of any of these three components by PMA could lead to an attenuation of VIP-stimulated adenylate cyclase activity. Several lines of evidence establish that PMA produces its effects at the level of VIP receptors. (i) Cholera toxin is known to stimulate cAMP production by promoting ADP-ribosylation of the α-subunit of N_s [10]. Similarly, F^- has been reported to activate adenylate cyclase through an interaction with N_s [10]. In the present study, PMA failed to attenuate the action of these compounds. (ii) Forskolin stimulates adenylate cyclase activity in intact cells or cell membrane preparations by interacting directly with the catalytic unit [11]. Again, PMA did not inhibit forskolin-stimulated cAMP production in HT29 cells. (iii) Finally, treatment of HT29 cells with PMA resulted in a 50% loss of VIP receptors as measured in cell membranes using ^{125}I-VIP. Altogether, these data indicate that the ability of PMA to decrease VIP-induced stimulation of adenylate cyclase does not involve an effect of the phorbol ester on either N_s or the catalytic unit. Rather, they point to a PMA-initiated process that leads to VIP receptor down-regulation, which in turn decreases VIP responsiveness in HT29 cells.

The tumor-promoting phorbol esters such as PMA are thought to exert their effect through activation of protein kinase C [4]. Phorbol esters can substitute for diacylglycerol, the physiological activator of protein kinase C produced from receptor-mediated breakdown of phosphatidylinositol 4,5-bisphosphate [12]. Recently, phorbol esters were reported to promote phosphorylation of insulin [13], epidermal growth factor [14,15] and β-adrenergic [16,17] receptors. In the latter case, phosphorylation resulted in loss of adenylate cyclase sensitivity to β-adrenergic agonists [16,17]. It is therefore tempting to speculate that in HT29 cells, PMA, through activation of protein kinase C, induces the phosphorylation of VIP receptors, which results in the inability of the phosphorylated receptors to bind VIP, and consequently in a decreased sensitivity of HT29 cells to the peptide.

Whether or not this represents the actual mechanism that leads to a loss of VIP binding in HT29 cells, the present data point to a possible role for protein kinase C in regulating VIP receptor function.

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