

Vasoactive intestinal peptide receptor activity and specificity during enterocyte-like differentiation and retrodifferentiation of the human colonic cancerous subclone HT29-18

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Commitment of HT29-18 cells to enterocyte-like differentiation by glucose removal is related to a decreased capacity to generate cAMP after treatment with vasoactive intestinal peptide (VIP), forskolin or sodium fluoride. In contrast, the potency of VIP ($EC_{50} = 1.1 - 1.3 \times 10^{-10}$ M) and the pharmacological specificity of the VIP receptor (VIP > rh GRF 1-43 > PHI > secretin) are unchanged during differentiation and retrodifferentiation. These results indicate that disturbances in VIP receptor – post-receptor activity, involving cell surface VIP receptors, membrane and intracellular transducers of hormonal information, occur during enterocyte-like differentiation of the HT29-18 subclone.

HT29-18 subclone Enterocyte-like differentiation VIP receptor activity Adenylate cyclase Forskolin

1. INTRODUCTION

Villus formation in the stratified epithelium of the fetal human small intestine at 8–9 weeks gestation is followed by the morphological and biochemical differentiation of the absorptive cells at 9–10 weeks gestation [1–3]. Histogenesis and morphogenesis of the intestine coincides with the development of the highly organized microvillus structure at the apical cell surface and with the appearance of many brush border enzymes in the absorptive cells [1,2]. Several investigations have documented the biological and biochemical actions of the vasoactive intestinal peptide (VIP) in the mature intestine [4]. They include net fluid and electrolyte secretions [5–7], receptor binding and adenylate cyclase activation in normal [8,9] and cancerous HT-29 intestinal cells [10]. We have characterized specific and functional VIP receptors in small intestinal epithelial cells isolated from human fetuses at 18–23 weeks gestation [11], and in intestinal villi isolated from rat fetuses at 19

days gestation [12,13]. At this stage of development, rat intestine exhibits an antero-posterior gradient of differentiation; thus, villus structures are well organized in the duodenum, and the lower part of the small intestine exhibited epithelial projections and a linear basal membrane [14].

For further investigation of plasma membrane development and cell surface receptor expression during the maturation of intestinal cells, [11–13] we evaluated VIP receptor activity during enterocyte-like differentiation and retrodifferentiation of the HT29-18 human colonic cancerous subclone [15]. These cultured clonal cells can be primed for differentiation into enterocyte-like cells in glucose-free medium containing 5 mM galactose (glucose removal) and dedifferentiated following subsequent substitution of galactose for glucose, as described in the noncloned HT-29 cell line [16]. Here we therefore measured VIP receptor activity in clonal and nonclonal HT-29 cells (i.e. cell cAMP generation and adenylate cyclase activation) and examined by electron microscopy the ultrastruc-

tural characteristics of HT29-18 cells cultured in a medium containing glucose or galactose. VIP receptor specificity was evaluated in undifferentiated and enterocyte-like HT29-18 cells incubated in the presence of VIP and of its 5 natural analogues: secretin, pancreatic glucagon, peptide having N-terminal histidine and C-terminal isoleucine amide (PHI), rat hypothalamic growth hormone releasing factor 1-43 (rh GRF 1-43) and gastric inhibitory peptide (GIP). Results were compared to those obtained with the ubiquitous adenylate cyclase activators sodium fluoride and forskolin. Part of this work has been published as an abstract [17].

2. MATERIALS AND METHODS

2.1. Cell culture

The undifferentiated and differentiated HT29-18 human colonic cancerous subclone, generously provided by Dr D. Louvard (Unité de Biologie des Membranes, Institut Pasteur, 75724 Paris) was cultured in Dulbecco's modified Eagle medium supplemented with either 25 mM D-glucose (undifferentiated cells) or 5 mM D-galactose (differentiated cells), plus 10% dialyzed fetal bovine serum, as described in [15,16]. Differentiation of HT29-18 cells was induced by gradual substitution of glucose for galactose in the culture medium by a daily increment of 20% for 5 consecutive days. Thereafter, cells were passaged 25 times and maintained in galactose-rich medium for 1 year. Retro-differentiation was induced by returning the enterocyte-like HT29-18 cells to glucose medium for 5 days. Cells were then passaged at a starting density of 6×10^6 cells per 75 cm² plastic flask and cultured for 1-2 weeks in 25 mM glucose-rich medium before experimentation. The glucose-rich medium was changed daily, and the galactose-rich medium, every 48 h.

The HT-29 cell line was kindly provided by Dr J. Fogh (Sloan Kettering Institute, NY) and cultured by the standard method described [18]. Between passages 26 and 34 in glucose-rich medium and between passages 30 and 36 in galactose-rich medium, HT29-18 cells were washed 3 times at 37°C with magnesium and calcium-free phosphate-buffered saline and removed from culture flasks after exposure to 0.02% EDTA. The isolated cells were washed 3 times at 20°C with 40 ml Krebs-

Ringer phosphate buffer (pH 7.4) and resuspended after centrifugation at $200 \times g$ for 3 min in 35 mM Tris-HCl buffer containing 1.4% bovine serum albumin (BSA) and 50 mM NaCl, (pH 7.5). Cell viability determined by trypan blue exclusion was about 80-95%.

2.2. Cell morphology

For ultrastructural examination, HT29-18 cells were harvested from culture flasks with a rubber policeman and fixed for 1 h at 4°C in 2% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). This material was then rinsed overnight in the same buffer and postfixed in 1% osmium tetroxide for 3 min at room temperature [19]. The pellet was then dehydrated and embedded in Epon 812. Ultrathin sections were obtained on an ultracut Reichert microtome. After double staining with uranyl acetate and lead citrate, the sections were examined in a Zeiss Em 109 electron microscope operated at 55 kV.

2.3. Cellular cAMP generation

In a standard assay, 150 μ l of the cell suspension (0.2 - 0.5×10^6 cells) was preincubated for 10 min at 37°C with or without the cAMP phosphodiesterase inhibitor isobutylmethylxanthine (IBMX) (1.2 mM), and in 250 μ l Tris-HCl buffer containing 1.4% BSA and 50 mM NaCl (pH 7.5). The reaction was initiated by adding 100 μ l of appropriate hormones or chemicals, and was stopped at the time indicated by adding 50 μ l HClO₄. cAMP was determined by the radioimmunoassay method detailed in [20]. The absolute values are expressed as pmol cAMP produced by 10^6 clonal and non-clonal HT-29 cells.

2.4. Adenylate cyclase preparation and assay

Undifferentiated and enterocyte-like HT29-18 cells were disrupted at 4°C by 3 bursts of 10 s each (15×10^6 cells/ml) with a Polytron homogenizer (Kinematica, Luzern, Switzerland) in 10 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA, 30 mM NaCl, 1 mM dithiothreitol and 5 μ M PMSF. The crude plasma membrane preparation was obtained by centrifugation ($27\,000 \times g$, 30 min at 4°C) in a Sorvall centrifuge RC-2 (Newton, CT). Adenylate cyclase activity was measured just after centrifugation by a modified version of the method in [11]. The reaction was initiated by addition of

the membrane-bound adenylate cyclase (15–20 μ g membrane protein per tube) and the mixture incubated at 30°C for 10 min. Data are expressed in pmol cAMP produced/min per mg membrane protein.

2.5. Expression of results

Results are presented as means $\pm$ SE. The significance of the differences observed was assessed by Student's *t*-test. Regression lines were fitted to the linear portions of the dose-response curves derived from individual experiments. The apparent EC₅₀ potencies, i.e. the doses required to produce half-maximal stimulation by the peptides, were calculated by the least square method. All calculations were performed on an HP-85 microcomputer (Hewlett-Packard).

2.6. Peptides and chemicals

Highly purified natural porcine VIP and PHI were purchased from Professor V. Mutt (GHI Laboratory, Stockholm, Sweden). Crystallized, highly purified porcine glucagon (lot 42306) was from Novo Research Institute (Bagsvaerd, Den-

mark). Synthetic porcine secretin and rat hypothalamic GRF 1–43 were prepared by Professor E. Wunsch (Max Planck Institut für Peptidchemie, Martinsried, FRG) and Dr J. Rivier and Dr W. Vale (Salk Institute, San Diego, CA) respectively. Forskolin, sodium fluoride, dithiothreitol, phenylmethyl sulfonyl fluoride (PMSF), GTP, pure ATP, cAMP and IBMX were from Sigma (St. Louis, MO). Na¹²⁵I was from the Radiochemical Centre (Amersham, England).

3. RESULTS

3.1. Morphological characteristics of HT29-18 cells

As shown in fig.1 (left), undifferentiated HT29-18 cells grow as disorganized multilayers and display an unpolarized nucleus as well as cytoplasmic projections randomly dispersed all round the cell periphery. Enterocytic-like HT29-18 cells have a reduced nuclear/cytoplasmic ratio and exhibit typical brush border structures composed of microvilli at their apical surface (fig.1, middle). The microfilaments of the microvillus core extend deep into the cytoplasm (fig.1, right). In these

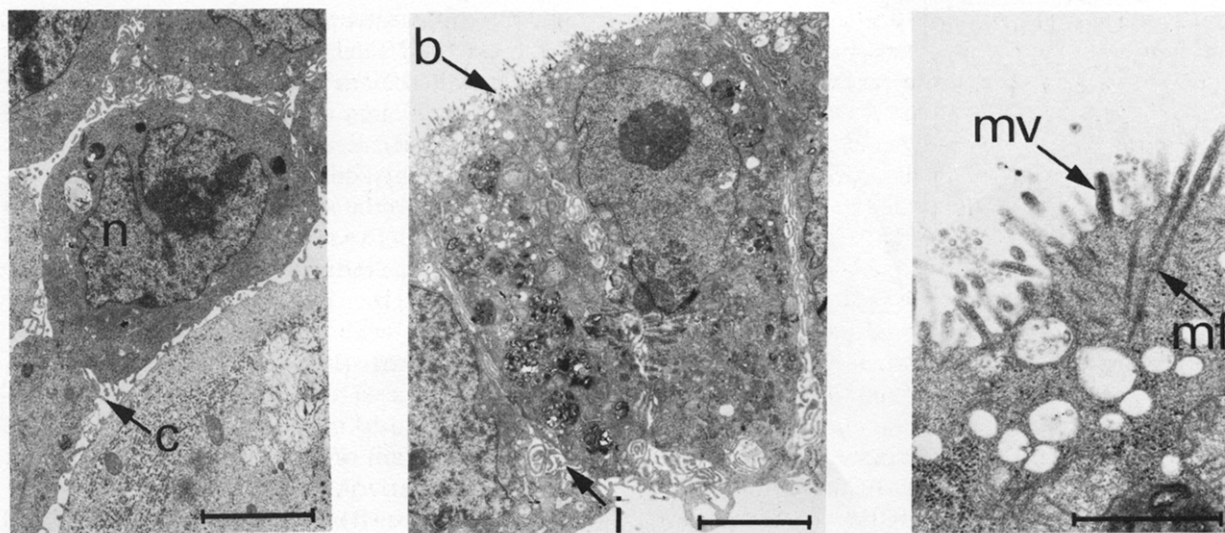


Fig.1. Ultrastructural appearance of undifferentiated and enterocyte-like HT29-18 cells. Left panel: transmission electron microscopy of undifferentiated cells growing as poorly organized epithelial layers in culture flasks. The population doubling time was 19 h in glucose-rich medium (n, nucleus; c, cytoplasmic projections; bar, 5 μ M). Middle panel: transmission electron microscopy of enterocyte-like cells growing as polarized monolayers. The population doubling time was 52 h in galactose-rich medium (b, brush border; i, interdigitations, bar, 5 μ M). Right panel: enlargement of the microvillus structure located at the apical surface of enterocyte-like cells (mf, microfilaments; mv, microvillus; bar, 1 μ m).

cells, apical junctional complexes, including tight junctions and desmosomes, have often been observed [17].

3.2. Kinetics of VIP receptor activity

As shown in fig.2 (expts 1 and 2), cellular cAMP levels were 1.4 ± 0.1 and 2.3 ± 0.3 pmol/ 10^6 cells in nonclonal and clonal HT-29 cells, respectively, incubated for 10 min at 37°C , in the absence of the phosphodiesterase inhibitor IBMX ($n=16-24$ determinations). After the addition of 10^{-8} M VIP, the concentration of cAMP rose within 30 s;

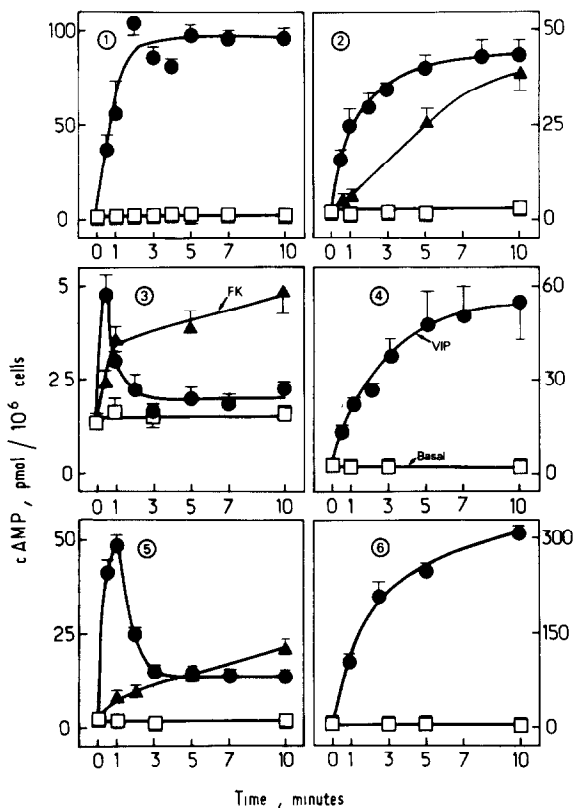


Fig.2. Effect of time and IBMX on VIP receptor activity in HT-29 cells and in the HT29-18 subclone cultured in glucose- or galactose-rich medium. Cyclic AMP generation was evaluated under basal ($\square$) or stimulated conditions [10^{-8} M VIP ($\bullet$) and 2×10^{-5} M forskolin ($\blacktriangle$)] in cells incubated at 37°C , either in the absence of IBMX (expt 1, noncloned HT-29 cells; expt 2, undifferentiated HT29-18 cells; expt 3, enterocyte-like cells, and expt 5, retrodifferentiated cells), or in the presence of 1.2 mM IBMX (expt 4, enterocyte-like cells, and expt 6, retrodifferentiated cells). Data are means $\pm$ SE from 3-5 experiments performed in duplicate or triplicate.

half-maximal and maximal stimulations were respectively observed within 45 s and 2 min in HT-29 cells and within 60 s and 5 min in the HT29-18 subclone. Maximal cAMP stimulation by VIP over basal levels represented a 70-fold increase (98 ± 7.5 pmol/ 10^6 cells) and a 17-fold increase (40 ± 3.6 pmol/ 10^6 cells) in HT-29 and HT29-18 cells respectively. In both types of cells, basal and VIP-stimulated cAMP levels remained constant for the remainder of the 10 min incubation. A very different picture was seen in the enterocytic-like cells (fig.2, expt 3). In this system, VIP produced a 3-fold increase in cAMP generation after 30 s (from 1.5 ± 0.1 to 4.8 ± 0.5 pmol/ 10^6 cells), followed by a rapid decline in cAMP levels which returned to basal values at 2 min. cAMP production (4.8 ± 0.6 pmol/ 10^6 cells) remained far above the basal level for 10 min. However, in the presence of 2×10^{-5} M forskolin, in retrodifferentiated cells (fig.2, expt 5), cAMP levels reached a peak value at 1 min in the presence of VIP, rising from 1.9 ± 0.3 to 48 ± 2.8 pmol/ 10^6 cells (i.e. a 25-fold increase), decreased to 15 ± 0.5 pmol at 3 min, and remained far above the basal level for the subsequent 7 min of incubation.

Similarly, after forskolin treatment, erythrocytic-like differentiation and retrodifferentiation of the HT29-18 subclone was associated with reduced cAMP accumulation. In HT29-18 cells incubated for 10 min at 37°C in the absence of IBMX, the cAMP levels measured in the presence of 2×10^{-5} M forskolin declined from 40 ± 2 pmol in the undifferentiated cells (expt 2) to 4.8 ± 0.6 pmol in the enterocyte-like cells (expt 3), and to 21 ± 3 pmol in the retrodifferentiated cells (expt 5).

Addition of the phosphodiesterase inhibitor IBMX during incubation of differentiated and retrodifferentiated HT29-18 cells fully restored (expt 4) or increased (expt 6) the cAMP production capacity previously measured in undifferentiated cells after addition of VIP (expts 1 and 2). Under these conditions, cAMP levels in differentiated HT29-18 cells rose from the basal levels of 1.8 ± 0.2 to 50 ± 12 pmol/ 10^6 cells (27-fold increase, expt 4), and in retrodifferentiated cells from 3.78 ± 0.3 to 310 ± 27 pmol/ 10^6 cells (80-fold increase, expt 6).

3.3. Adenylate cyclase activity

After the addition of 10^{-8} M VIP, adenylate cyclase activity in undifferentiated HT29-18 cells

Table 1

Effect of VIP and sodium fluoride on adenylate cyclase activity in undifferentiated and enterocytic-like HT29-18 cells

Substance	Dose	Adenylate cyclase activity			
		Undifferentiated cells		Enterocyte-like cells	
		pmol/min per mg protein	S - B/B	pmol/min per mg protein	S - B/B
None	—	55 ± 8		260 ± 60	
NaF	10 ⁻² M	645 ± 130	10.7 ± 1.2	1745 ± 400	5.7 ± 1.2*
VIP	10 ⁻⁸ M	365 ± 80	5.7 ± 1	770 ± 210	1.9 ± 0.5*

Absolute values (pmol/min per mg protein) and average -fold stimulation over basal adenylate cyclase activity (S - B/B) are means ± SE from 5 (differentiated HT29-18 cells) to 6 different membrane preparations (undifferentiated HT29-18 cells). Each assay was performed in duplicate. Significantly different at $p < 0.05^*$ from the corresponding control value

increased from 55 ± 8 to 365 ± 80 pmol/min per mg protein i.e. a 5.7-fold rise in relation to the paired basal values (table 1). In enterocyte-like cells, the average stimulation by VIP dropped to a factor of 1.9 ± 0.5 . The same applied to adenylate cyclase stimulation by fluoride in undifferentiated and enterocyte-like cells, i.e., 10.7-fold vs 5.7-fold increase.

3.4. Potency of VIP and pharmacological specificity of the VIP receptor

The pharmacological specificity of the VIP receptor during enterocyte-like differentiation and retrodifferentiation of the HT29-18 subclone was established towards the relative potencies of VP and of its natural analogues as follows: VIP > rh GRF 1-43 > PHI > secretin (fig.3). Glucagon and

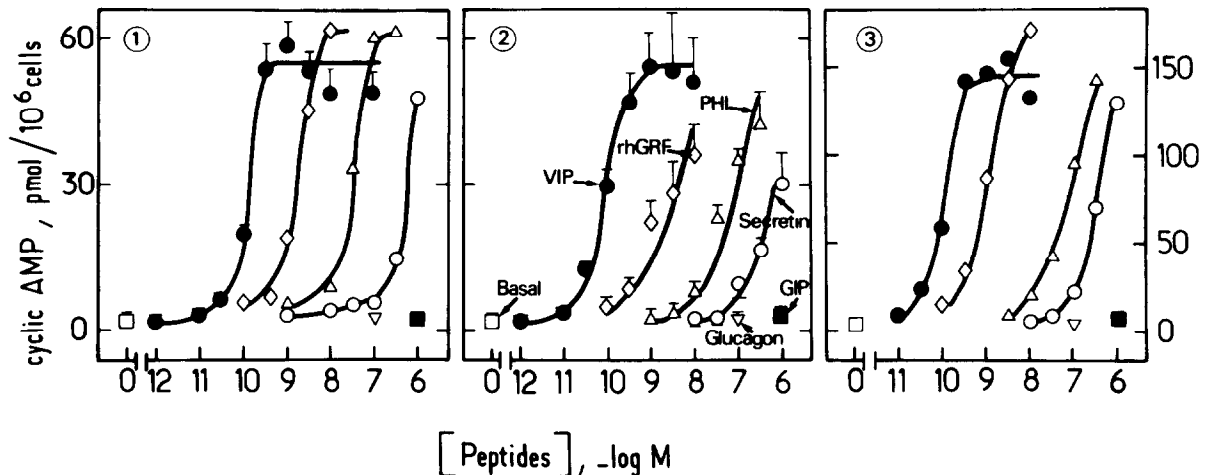


Fig.3. Pharmacological specificity of VIP receptor activity during enterocyte-like differentiation and retrodifferentiation of the HT29-18 subclone. Undifferentiated (expt 1), differentiated (expt 2) and retrodifferentiated cells (expt 3) were incubated at 37°C for 5 min in the presence of 1.2 mM IBMX and of various concentrations of VIP (●) and of its analogues: rh GRF 1-43 (◇), PHI (Δ), secretin (○), pancreatic glucagon (▽) and GIP (■). Basal cAMP levels (□). Results are means ± SE from 3-6 experiments performed in duplicate (expts 1 and 2). Individual experiments from duplicate determinations are illustrated in expts 1 and 3.

GIP were inactive. Similarly, VIP potency in the 3 cell types was unchanged; at $1.3 \pm 0.3 \times 10^{-10}$ M in HT29-18 cells, $1.1 \pm 0.2 \times 10^{-10}$ M in enterocyte-like cells and 1.2×10^{-10} M VIP in retrodifferentiated HT29-18 cells.

4. DISCUSSION

The basic finding of this work indicates that enterocyte-like differentiation of the human colonic HT29-18 subclone corresponds to a decreased capacity of the differentiated cells to generate cAMP after forskolin treatment and cell surface receptor activation by VIP. We previously obtained very similar results in normal intestinal cells isolated from fetal and neonatal rats [12,17]. In this model, VIP produced marked persistent stimulation for 10 min in villous cells isolated from fetal rats at 19 days of gestation. This stimulation was transient in 5 day-old rats and undetectable in adults. Retrodifferentiation of HT29-18 enterocyte-like cells after substitution of galactose for glucose partly reversed this process, suggesting that retrodifferentiated cells return to an intermediary stage in their programme of differentiation and dedifferentiation induced by the glucose-galactose balance in the culture medium. Accordingly, Louvard et al. [15] previously observed that the biosynthesis and storage of villin was 10-times higher in enterocyte-like cells than in the undifferentiated HT29-18 subclone, whereas villin levels remained high in retrodifferentiated cells. Further, in noncloned HT-29 cells, Pinto et al. [16] showed that the differentiation process was reversible for maltase, sucrase and aminopeptidase activity but not for alkaline phosphatase which stayed high. In this work, electron microscope examination revealed that retrodifferentiated cells assume the morphological characteristics of partially dedifferentiated enterocyte-like HT29-18 cells (not shown). In contrast, the potency of VIP and the pharmacological properties of the VIP receptor were remarkably similar in all 3 cell types. The structural requirements of the VIP receptor for adenylate cyclase activation therefore remained unchanged during HT29-18 cell differentiation and retrodifferentiation. In this connection, we recently reported that VIP receptors in small intestine epithelial cells isolated from human fetuses at 18–23 weeks gestation and from rat fetuses at 19

days gestation are pharmacologically similar (VIP > PHI > secretin) to those characterized in mature intestine [11–13]. We also made the same observation [19] for the secretin receptor in fundic and antral glands isolated during rat development, from the fetal to adult age stages (secretin > PHI, VIP).

These variations in VIP receptor activity observed here during intestinal cell differentiation in the HT29-18 subclone and previously in the developing rat [12] might be due to changes in the following biochemical and physical parameters: the number, desensitization, internalization and turnover of VIP receptors, plasma membrane fluidity, adenylate cyclase pools, activity of the N_i and N_s subunits, cAMP phosphodiesterase activity (cAMP-PDE), or a combination of these parameters. In agreement with this hypothesis, we recently demonstrated that VIP can down-regulate its own receptors [21,22] after short- or long-term VIP treatment of noncloned HT-29 cells and HGT-1 human gastric cells (desensitization). The equilibrium conditions obtained here in undifferentiated cells after addition of VIP are due to the balance between cAMP generation and degradation (fig.2, expts 1 and 2). The finding that addition of IBMX fully restored VIP receptor activity suggests that cAMP-PDE activities changed during differentiation and retrodifferentiation. In this connection, Mangeat et al. [23] reported rapid specific activation within 1 min of the low K_m cAMP-PDE in noncloned HT-29 cells incubated at 37°C with VIP (10^{-10} – 10^{-8} M). From the comparative kinetic data for cAMP generation after VIP and forskolin, it is therefore likely that both receptor desensitization and cAMP-PDE activation coincide with the transient rise in cAMP levels evoked by VIP in enterocyte-like cells incubated in the absence of IBMX (fig.2, expt.3). Secondly, it has been shown that the physical state of the plasma membrane (lipid microviscosity) may promote inhibition or stimulation by a hormone via either the N_i or the N_s structures of the cyclase [24]. For example, Robberecht et al. [25] have shown that short-chain alcohols inhibited forskolin-stimulated rat cardiac adenylate cyclase, probably by interacting with lipids and altering membrane fluidity. It has been proposed in different systems that forskolin acts directly on the catalytic unit C of the cyclase and also on the

mechanisms coupling this unit with the guanine nucleotide sites N_s and N_i [26-29]. In the case of the intestine, changes in lipid composition and membrane fluidity have been reported in the developing rat [30] and rabbit [31]. It is therefore tempting to speculate that enterocyte-like differentiation of the HT29-18 clone might be connected with some functional defect between the catalytic subunit and its regulatory proteins. In agreement with this proposition, we have demonstrated here that the extent of stimulation of adenylate cyclase activity after VIP and sodium fluoride dropped appreciably after enterocyte-like differentiation of the HT29-18 subclone.

Decreased membrane fluidity during differentiation of human promyelocytic leukemia HL-60 cells [32] by dimethyl sulfoxide is also associated with reduced capacity of the differentiated granulocytic-like cells to generate cAMP after histamine H_2 receptor activation [33]. The same observation was made during retinoic acid-induced monocytic differentiation of the human histiocytic lymphoma cell line U-937 [34,35].

Taken together, the present results and those for our studies of rat intestinal development [12,13] show that VIP receptor activity is indeed an indicator of intestinal cell maturation. Additional studies are needed to characterize further the factor(s) responsible for the disturbing effects of enterocyte-like cell differentiation on VIP receptor-adenylate cyclase activities, and to assess their impact on the normal and tumoral development of the intestine.

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