The purpose of this work was to study vasoactive intestinal peptide (VIP) receptors and the adenylate cyclase response to VIP upon enterocytic differentiation of the human colon adenocarcinoma cell line Caco-2 in culture. The VIP-stimulated enzyme activity is very low, e.g. 20% above basal activity in undifferentiated cells (day 5) and is enhanced markedly at confluence reaching a maximum, e.g. 270%, above basal activity in fully differentiated cells (day 30). VIP potency is also slightly enhanced, the EC<sub>50</sub> of VIP ranging from 0.31 nM at day 5 to 0.07 nM at day 30. Modifications of the adenylate cyclase system are not responsible for the development of VIP response. Indeed, forskolin-stimulated adenylate cyclase activity is unchanged during differentiation supporting no alteration of the enzyme catalytic subunit. The same holds true for NaF and guanosine 5'-β,γ-imidodiphosphate, indicating a constant activity of the guanine nucleotide regulatory unit which mediates hormonal stimulation of adenylate cyclase (N<sub>s</sub>). This is further supported by the similar extent of cholera toxin-catalyzed [32P]ADP-ribosylation of the N<sub>s</sub> protein that is observed during differentiation. In sharp contrast, a dramatic increase of VIP receptor concentration is observed ranging from 32 fmol/mg of protein at day 5 to 414 fmol/mg of protein at day 30. This is confirmed by affinity cross-linking experiments showing an increased specific incorporation of <sup>125</sup>I-VIP in a major 66,000-dalton component during differentiation. A slight increase in receptor affinity is also observed during differentiation with K<sub>d</sub> ranging from 0.39 nM at day 5 to 0.08 nM at day 30.

These data indicate that one population of VIP receptors accumulates during Caco-2 cell differentiation, representing the crucial event in the development of adenylate cyclase response to the peptide.

VIP<sup>1</sup> receptors are present in a variety of tissues in consonance with the ubiquitous distribution of VIP in the central and peripheral nervous systems (1). Biochemical and membrane fusion techniques have shown that the VIP-sensitive adenylate cyclase is composed of three distinct components including the VIP receptor, the N<sub>s</sub>, GTP-binding protein, and the catalytic moiety of adenylate cyclase (2-4). However, very little is known about how these components are integrated during cell differentiation. The intestinal epithelium offers a good system to address this question for several reasons. First, VIP receptors have been extensively described in this tissue where VIP promotes the secretion of water and ions through a cyclic AMP-dependent pathway (reviewed in Ref. 5). Second, intestinal epithelial cells differentiate rapidly from stem cells into mature enterocytes in vivo with a gradient of differentiation along the crypt-villus axis (reviewed in Ref. 6). Third, some cell lines derived from human colon cancer cells have been shown to undergo partial or terminal enterocytic differentiation in vitro (reviewed in Ref. 7). In particular, the Caco-2 cell line established by Fogh et al. (8) from a human colon adenocarcinoma develops in culture a number of differentiated functions characteristic of enterocytes as recognized a few years ago by Pinto et al. (9). This differentiation is a growth-related process starting after confluence (9, 10) and characterized by the presence of tight junctions, regular apical brush-border microvilli with associated hydrolases, formation of domes (9, 10), and epithelial electrical properties (11, 12) unique of transporting epithelia. Confluent Caco-2 cells catalyze active vectorial transport of fluid and electrolytes across the cell monolayer, a process regulated by cyclic AMP and VIP (11, 12).

We recognized early that Caco-2 cells were equipped with VIP receptors (13) and recently noted that the enterocytic differentiation of Caco-2 cells is accompanied with an enhanced response of adenylate cyclase to VIP. In the present study, we document the development of VIP-sensitive adenylate cyclase in this model and address the question of what components of the receptor-coupled enzyme are responsible for this phenomenon. We provide evidence that the adenylate cyclase response to VIP develops parallel to other differentiated functions of Caco-2 cells and that the increased response is due to an increase of VIP receptor level.

**EXPERIMENTAL PROCEDURES**

**Materials**—Purified porcine VIP was provided by Dr. V. Mutt (Karolinska Institute, Stockholm, Sweden), synthetic VIP 2-28 by Profs. St. Pierre (University of Sherbrooke, Quebec, Canada), synthetic peptide histidine isoleucineamide and secretin by Prof. L. Moroder (Max-Planck Institute, Martinsried, West Germany) and synthetic human growth hormone-releasing factor 44-NH<sub>2</sub> by Profs.

<sup>1</sup> The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

<sup>2</sup> The abbreviations used are: VIP, vasoactive intestinal peptide; N<sub>s</sub>, the guanine nucleotide regulatory unit which mediates hormonal stimulation of adenylate cyclase; Gpp(NH)p, guanosine 5'-β,γ-imidodiphosphate; SDS, sodium dodecyl sulfate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

* A preliminary account of this investigation was presented at the Federation of American Societies for Experimental Biology Summer Research Conference on Gastrointestinal Differentiation, Saxton's River, VT, July 21-26, 1985.
VIP Receptor Development in Caco-2 Cells

R. GUILLEMIN AND N. LING (The Salk Institute for Biological Studies, San Diego, CA). 

125I-VIP was prepared by the chloramine-T method at a specific activity of 750 Ci/mmol as described (14). It displayed the same activity as native VIP in stimulating cyclic AMP production in the cultured cell line HT-29 (15). Chemicals and unstained protein markers for SDS-gel electrophoresis were purchased from Bethesda Research Laboratories, [3H]GTP (N. Edwards) from Du Pont-New England Nuclear and dithiobis-(succinimidyl propionate) from Pierce. Other chemicals of highest purity commercially available were obtained from Sigma.

Cell Culture—The cell line Caco-2 was obtained from J. FOGH (Memorial Sloan-Kettering Cancer Center, Rye, NY). The cells were cultured as previously reported (10) and used between passages 40 and 70. It was verified that the cell volume and the quantity of protein per cell do not vary significantly upon cell growth. Indeed, the cell volume was 5.85 x 10^-6 and 7.95 x 10^-6 μL and the quantity of protein per cell was 0.49 and 0.48 ng in day 5 and day 25 cells, respectively.

Membrane Preparation—A particulate fraction from cell homogenate was obtained exactly as described (16). This preparation was shown previously to be adequate for measuring VIP receptors (16) and adenylate cyclase activity (10) in cultured human colon adenocarcinoma cell lines Caco-2 and HT-29.

125I-VIP Assays and Electron Microscopy—Enzyme activities were measured in the same membrane preparations (about 100 μg of protein/ml) as those used for receptor studies. Sucrase (EC 3.2.1.48), aminopeptidase N (EC 3.4.11.2), and dipeptidyl peptidase IV (EC 3.4.14.5) activities were determined as described in Refs. 17-19, respectively. The enzyme activities are expressed as milliunits per mg of protein, where a milliunit is defined as the activity which hydrolyzes 1 μmol of substrate per min at 37 °C.

Transmission electron microscopy was performed on day 5 or 25 on cells grown in 25-cm² plastic flasks as already described (10).

Binding of 125I-VIP to Receptors—Membranes (about 100 μg of protein/ml) were incubated as described (16) for 60 min at 30 °C with 125I-VIP (0.05 nM) and other compounds, when necessary, in 50 mM Hepes, pH 7.5, containing 2% (w/v) bovine serum albumin and 0.1% (w/v) bacitracin. The reaction was stopped and the radioactivity bound to membranes was measured by gamma spectrometry as described (15). Specific binding was calculated as the difference between the amount of 125I-VIP bound in the absence (total binding) and presence (nonsaturable binding) of 1 μM unlabeled VIP. Each binding measurement within one experiment was performed in duplicate.

Degradation of 125I-VIP—The integrity of 125I-VIP in the incubation medium after exposure to Caco-2 cell membranes from different days of culture was tested by its ability to rebinding to fresh membranes from day 25 cells as described (15).

Cross-linking of 125I-VIP to Receptors—Membranes (0.5 mg of protein/ml) were incubated as described above but in 5 ml of buffer containing 125I-VIP. The incubation was stopped by adding 35 ml of ice-cold 25 mM Hepes buffer, pH 7.5, and membrane-bound 125I-VIP was obtained by centrifugation for 10 min at 30,000 × g. The pellet was used immediately for subsequent cross-linking as described in detail (3). At the end of the incubation, the reaction was quenched and the membranes were pelleted as described (3). Membrane proteins were then analyzed by SDS-polyacrylamide gel electrophoresis according to the procedure of Laemmli (20). Samples were prepared and analyzed on a 5-15 or a 10% polyacrylamide slab gel with a 3% stacking gel as described previously in detail (3, 16).

Adenylate Cyclase Assay—Adenylate cyclase was assayed as described in detail elsewhere (21). The incubation medium contained 60 mM Hepes buffer, pH 7.5, 0.4% (w/v) bovine serum albumin, 0.1% (w/v) bacitracin, 0.3 mM ATP, 20 mM creatine phosphate, 1 mg/ml creatine kinase, 5 mM MgCl₂, 0.2 mM 3-isobutyl-1-methylxanthine and, unless otherwise stated, 10 μM GTP. Cyclic AMP was determined by radioimmunoassay as described (22).

Cholera Toxin-Catalyzed ADP-Ribosylation of Membranes—Cholera toxin was activated before use as described (4, 23). For ADP-ribosylation, membranes (0.4 mg of protein/ml) were incubated with activated toxin (50 μg/ml) and membrane proteins were subjected to SDS-polyacrylamide gel electrophoresis as described (4).

Protein Determination—Membrane proteins were determined by the method of Bradford (24) with bovine serum albumin as a standard.

RESULTS

Cell Growth and Differentiation—The proliferation of Caco-2 cells begins after a lag period of 48 h. Confluency is reached on day 6 and the stationary phase on day 9. During the phase of active growth (until day 6), no regular microvilli can be observed at the apical surface of the cells (Fig. 1, left), although the cells are already polarized. A typical apical brush border only appears after confluence (Fig. 1, right). The development of brush-border-associated hydrolase activities is also a process occurred after confluence, e.g. antigenic properties are low during the active phase of growth and increase regularly from day 10 onward, reaching on day 25 maximal values which are 20-fold the basal value for sucrase activity (165 million units/mg of protein) and twice the basal values for both aminopeptidase N and dipeptidyl peptidase IV activities (16 and 145 million units/mg of protein, respectively).

Development of VIP-stimulated Adenylate Cyclase—Activation of adenylate cyclase by VIP is very weak in membranes from day 5 cells and increases markedly during enterocytic differentiation (Fig. 2). VIP stimulates adenylate cyclase activity 270% at day 30 but elevates enzyme activity only 20% above basal activity at day 5. The major increase of VIP efficacy occurs after cells have reached confluence, e.g. after day 10, and is concomitant with the development of differentiated properties of Caco-2 cells, in particular hydrolytic enzyme activities. The potency of the peptide is also slightly enhanced during differentiation since the concentration of VIP eliciting half-maximal response (EC₅₀) ranges from 0.31 nM at day 5 to 0.67 nM at day 30 (Fig. 2 and see also Fig. 5). Since the development of VIP-responsive adenylate cyclase during enterocytic differentiation of Caco-2 cells could be due to the accumulation of VIP receptors, an increase in the level of the N, GTP-binding protein and catalytic subunit of adenylate cyclase, and/or changes in the coupling properties between these components, we have investigated these possibilities. The possible alteration of the N, protein or adenylate cyclase catalytic subunit was first tested. The ability of sodium fluoride and forskolin to activate adenylate cyclase is similar in undifferentiated and differentiated cells (Table 1), in sharp contrast with the increasing VIP effects. Since forskolin is believed to act directly upon the catalytic subunit of adenylate cyclase (25) and fluoride ion via the N, regulatory protein (26), these data suggest that the activity of each component is independent of the differentiation of Caco-2 cells. This is further supported by the identical dose responses of Gpp(NH)p in activating adenylate cyclase at days 5, 10, 15, and 25 (not shown). In order to gain more direct information on the N, protein during differentiation, membranes from cells collected at days 5, 15, and 25 have been treated with cholera toxin in the presence of [3H]NAD under conditions in which the N, protein is specifically labeled by ADP-ribosylation of its α-subunit (27-30). It appears that the cholera toxin-induced incorporation of 32P in the α-subunit of N, is similar at days 5, 15, and 25 (not shown), suggesting that the N, protein is present at the same level whether the cells are differentiated or not.

Development of VIP Receptor Sites—The study of the time course of 125I-VIP binding to membranes prepared from Caco-2 cells at days 5, 15, and 25 shows that the amount of tracer (0.05 nM) specifically bound at equilibrium increases markedly from day 5 (7 ± 4 fmol/mg of protein) to day 25 (90 ± 11 fmol/mg of protein). This is not related to changes in the ability of membranes to inactivate 125I-VIP during cell growth. Indeed, degradation of 125I-VIP is low (<20%) and does not vary during cell differentiation, with the exception of day 5 cell membranes, which do not inactivate 125I-VIP at all. Therefore, it may be assumed that the increase of 125I-VIP binding during differentiation is related to an increased receptor concentration and/or affinity. To document this point, VIP receptors have been compared in equilibrium binding studies.
Fig. 1. Transmission electron microscopy of pre-confluent (day 5) and post-confluent (day 25) Caco-2 cells. Sections are perpendicular to the bottom of the flasks (arrow). Day 5 cells are shown to the left (× 5800) and day 25 cells to the right (× 3200). Note the presence of irregular microvilli (imu) on the apical surface of exponentially growing cells (left) contrasting with the presence in stationary cultures (right) of regular brush-border microvilli (bbm).

Fig. 2. Dose response of VIP in stimulating adenylate cyclase activity during the growth in culture of Caco-2 cells. Adenylate cyclase assays were performed as described under “Experimental Procedures” on cell membranes from day 5 to day 30 cultures. Results are expressed as VIP-stimulated enzyme activity/basal enzyme activity and represent the mean ± S.E. of four experiments. Day of culture for each dose-response curve is indicated within circles. Basal enzyme activities are shown in Table I. Arrows indicate the concentration of VIP eliciting half-maximal stimulation of enzyme activity.

TABLE I
Adenylate cyclase response to fluoride and forskolin as a function of Caco-2 cell growth in culture

| Day of culture | Adenylate cyclase activity |
|---------------|---------------------------|
|               | Basal | NaF (10 mM) | Forskolin (0.1 mM) |
| 5             | 129 ± 34 | 1006 ± 206  | 3457 ± 413 |
| 15            | 94 ± 33  | 893 ± 91    | 2406 ± 376 |
| 25            | 111 ± 38 | 1082 ± 94   | 2997 ± 289 |

Capacity is very low at day 5 and reaches a 13-fold higher value at day 30. A slight increase of receptor affinity is also noted upon enterocytic differentiation (see also Fig. 5).

Further experiments are conducted to identify the VIP receptor at the molecular level during cell differentiation. For that purpose, the cross-linker dithiobis-(succinimidyl propionate) is used to covalently attach ¹²⁵I-VIP to receptor sites in membranes from day 5, 15, and 25 cells. The subsequent SDS-polyacrlyamide gel electrophoresis analysis of membrane proteins reveals a major band of M₉ 66,000 whose labeling intensity greatly increases from day 5 to day 25 (Fig. 4). The labeling of this band is specific in that it is abolished by 0.3 μM unlabeled VIP in the three conditions. This band is typical of the high affinity VIP receptor in human colon as previously characterized in normal intestine (31).
The peptide specificity of VIP receptors in Caco-2 cells is tested with some naturally occurring VIP related peptides such as human growth hormone-releasing factor (hGRF), peptide histidine isoleucineamide (PHI), and secretin and the synthetic fragment of VIP, VIP 2-28. The order of affinity of the different peptides for VIP receptors in day 25 cell membranes is VIP > VIP 2-28 > hGRF = PHI > secretin. Identical data are obtained with day 5 cell membranes (not shown). This peptide specificity is typical of the human intestinal VIP receptors (20, 32, 33).

**Correlation between the Development of VIP Receptor and VIP-responsive Adenylate Cyclase—**Since the VIP receptor appears to be the only component of the receptor-coupled adenylate cyclase system that increases during differentiation, we have tried to correlate the development of VIP receptor to that of VIP-responsive adenylate cyclase. When receptor concentration is plotted against the efficacy of VIP in stimulating adenylate cyclase activity upon differentiation, a close correlation is observed (Fig. 5, left). The adenylate cyclase response even appears to develop in direct proportion to the concentration of VIP receptor in Caco-2 cells. On the other hand, the coupling efficiency index, e.g., the Kᵦ/Kᵦᵦᵦ ratio (34), is not modified during differentiation (Fig. 5, right), further supporting that the development of the adenylate cyclase response to VIP is related to changes in VIP receptors and not to modifications of coupling properties between receptors and components of the adenylate cyclase system.

**DISCUSSION**

This study shows that the adenylate cyclase response to VIP develops during the enterocytic differentiation of Caco-2 cells in culture. Several observations suggest the crucial role of VIP receptors in the development of the response. Equilibrium binding studies, using ¹²⁵I-VIP, reveal a sharp increase in VIP receptor concentration during differentiation. This is further confirmed by cross-linking experiments showing that the covalent labeling of the 63,000-dalton receptor protein increases markedly from day 5 to day 25 of cell culture (see Fig. 4). The apparent molecular weight of this receptor protein, and its ability to discriminate between different VIP agonists in differentiated Caco-2 cells are identical to those observed in the normal counterpart of Caco-2 cells, e.g., human colonic epithelial cells (20, 31-33). On the other hand, our observations indicate no major modification of the N₆GTP regulatory protein, the activity of the catalytic subunit of adenylate cyclase, or coupling properties of receptor, Nᵦᵦᵦ, and enzyme. Therefore, the increased receptor level appears to account entirely for the development of the VIP-stimulated adenylate cyclase activity. The close correlation between receptor level and VIP efficacy in stimulating enzyme activity during differentiation (see Fig. 5) also emphasizes this view. Moreover, this is confirmed by the fact that the slight increase of receptor affinity for VIP upon differentiation is directly reflected in an increase of VIP potency in stimulating cyclase activity (see Fig. 5). The increased affinity in the stimulation of adenylate cyclase may be related to the presence of so-
called “spare VIP receptors” (5) and/or to changes in the composition of lipids and in membrane fluidity in the plasma membrane during cell growth (34–37) and differentiation (38), inasmuch as VIP receptor activity is dependent on its phospholipidic environment (39). Finally, our results also argue for the absence of stoichiometric limitation in the number of regulatory components, e.g. the N protein, and the number of catalytic components, e.g. adenylate cyclase, during the development of VIP receptor-mediated cyclase response.

The present work shows that the development of sensitivity to VIP upon enterocytic differentiation of Caco-2 cells is due to an increase of receptor level. Similar observations have been made for the differentiation-related development of acetylcholine sensitivity in BChE muscle cells (40) or insulin sensitivity in 3T3-L1 adipocytes (41). However, the acquisition of cellular sensitivity to its neuroendocrine environment may also arise from other mechanisms. For example, in the 3T3-L1 model, a sharp decline of GTP-stimulated adenylate cyclase activity precedes the manifestation of catecholamine-stimulated enzyme activity during differentiation of preadipocytes, suggesting that a modulation of the levels and/or properties of the N2 GTP regulatory protein is involved (42). Likewise, induction of catecholamine-responsive adenylate cyclase in HeLa cells by sodium butyrate is related to qualitative changes in the regulatory component that facilitates its ability to couple to receptors (43). Finally, during the biological maturation of the rabbit heart, the β-adrenergic receptor, the GTP-dependent transduction event, and the catalytic subunit of adenylate cyclase exhibit significant developmental changes (34).

The development of VIP receptors in differentiating Caco-2 cells is concomitant with the morphological and functional differentiation of these cells. This is particularly obvious from the study of brush-border enzyme activities and the evolution of dome formation (9, 10). Like the functional and enzymic differentiation of Caco-2 cells (9–11), the accumulation of VIP receptors is a growth-related phenomenon. The levels of VIP receptors as well as the levels of aminopeptidase, alkaline phosphatase, and more particularly sucrase (9, 10) are low during the phase of active growth and start to increase when the cells stop growing. This sequence also applies to the formation of domes, which are indicative of transporting polarized cell monolayers (9). The growth-related differentiation of cultured Caco-2 cells is similar to the situation found in the normal intestine, where dividing crypt cells are undifferentiated and undergo differentiation when they migrate onto the villi and stop to divide (6). In that respect, it is of interest to note that the concentration of VIP receptors in differentiated Caco-2 cells is similar to that previously determined in mature epithelial cells from human colon (44) and rat small intestine (45).

The Caco-2 cell line offers an invaluable model to document the mechanism whereby the number of functional VIP receptors is controlled during cell differentiation. Such data are not available for any adenylate cyclase-coupled receptors. However, the absence of convenient tools such as cDNA probe for the VIP receptor or anti-VIP receptor antibodies makes it difficult to analyze this mechanism further at the present time. These tools should be developed in a few years owing to the recent solubilization of functional VIP receptors from plasma membranes (4).