Vasoactive Intestinal Peptide Receptor Regulation and Reversible Desensitization in Human Colonic Carcinoma Cells in Culture

C. Boissard, J-C. Marie, G. Hejblum, C. Gespach, and G. Rosselin

Unité de Recherches sur les Peptides Neurodigestifs et le Diabète, U55, INSERM, Centre de Recherches Saint-Antoine, 184, rue du Faubourg Saint-Antoine, 75571 Paris Cedex 12, France

ABSTRACT

Vasoactive intestinal peptide (VIP) receptors are widely distributed in different tissues or carcinoma cells originating from endoderm and have been shown to regulate the growth of colonic adenocarcinoma cells through the action of cyclic AMP (cAMP). After exposure of cultured HT-29 human colonic carcinoma cells to 10^{-8} M VIP, the cAMP-mediated signals in response to a new challenge with this neuropeptide were strongly attenuated as a function of time (half-life, <3 min) and VIP concentrations (half-maximal desensitization, 4 × 10^{-9} M VIP). Desensitization is receptor mediated as indicated by: (a) the pharmacological specificity of the desensitization (VIP > secretin); (b) the considerable decrease of the potentiative action of VIP on forskolin-induced cAMP generation; and (c) the close temporal relationship between VIP receptor desensitization and the disappearance of the VIP binding sites from the cell surface. Desensitization is reversible upon the removal of VIP. Recovery of functional VIP receptors is insensitive to cycloheximide treatment, is critically dependent upon temperature, and in optimal conditions (37°C) does not exceed 75 and 55% of the binding of 125I-VIP monoiodinated on tyrosine residue and VIP-induced cAMP production, respectively. The characteristics of the desensitization and internalization/recycling of the VIP receptors in carcinoma cells in culture are consistent with the transient action of this neurotransmitter and underlie the biological significance of these processes. The study of drugs and natural agents interfering with membrane regulation of VIP receptor density and activity may be of considerable importance in intestinal cell tumor biology.

INTRODUCTION

VIP composed of 28 amino acids is a multifunctional neurotransmitter (1, 2). It belongs to the secretin family of peptides, since porcine VIP and secretin share 14 amino acid identities in their primary sequence (1). VIP acts through specific binding sites coupled to adenylate cyclase and cAMP-dependent protein kinase activations (3–10). VIP receptors are widely distributed in different tissues or carcinoma cells originating from the entoderm, including the intestine (11–13), the stomach (14), the larynx (15), and the lung (16), and are also present in prolactinoma (17), GH3 cells (18), pancreatic adenocarcinoma (19), osteosarcoma (20), and lymphoblastic cells (21). In normal epithelium, VIP receptors mediate the hydro-ionic transfer in the small intestine (22) and the colon (23). In human HT-29 colonic carcinoma cells, VIP retains its ability to stimulate ionic exchange (24) and glycogenolysis (25). VIP has also been shown to suppress DNA synthesis in rat colonic mucosa by raising cAMP levels (26). Dibutyryl cAMP also inhibits cell proliferation in colonic crypts and colonic adenocarcinoma in rodents (27). However, dibutyryl cAMP at high doses inhibits crypt proliferation in rat jejunum, whereas lower doses accelerate crypt cell proliferation, indicating the necessity of detailed investigations and careful interpretations of data dealing with the regulation of cAMP levels during growth and differentiation of gut epithelial cells.

Our research has recently shown that the transient effect of VIP in activating phosphorylase and stimulating glycogenolysis in the HT-29 human colonic carcinoma cell line was correlated with a considerable and specific diminution of VIP-stimulated cAMP levels following previous exposure of the cells to VIP (28). The possibility of a rapid internalization of VIP has been shown in HT-29 cells (28–30). Moreover, we report here the analysis of the molecular mechanisms involved in the modulation of the VIP signals at the HT-29 cell surface. We therefore examined the kinetics and stoichiometry of the desensitization to VIP and internalization of VIP receptors in HT-29 cells. The receptor specificity and the molecular basis of the VIP desensitization process are investigated in relation to the actions of the VIP receptor agonist secretin and the dieterpine forskolin, known to activate directly adenylate cyclase in intact cells (reviewed in Ref. 31). The reversibility of the desensitization was also investigated with reference to the compartmental partition of the intracellular and cell surface VIP binding sites.

MATERIALS AND METHODS

Hormones and Chemicals. Natural porcine VIP was supplied by V. Mutt (Stockholm, Sweden). Synthetic porcine secretin was prepared and generously donated by E. Wünsch (Munich, West Germany). VIP was iodinated by the chloramine T method and purified by HPLC, as previously described (32). 125I-VIP, with a specific activity of 2000 Ci/mmol, was used in all experiments and has a biological activity similar to native VIP (33). Forskolin was obtained from France Biochem (Calbiochem, Meudon, France). The phosphodiesterase inhibitor IBMX was purchased from Sigma Chemical Company. All other agents were of analytical grade.

Colonic Carcinoma Epithelial Cells in Culture. The human colonic carcinoma cell line HT-29 was generously donated by J. Fogh (Sloan Kettering Institute, Rye, NY). The HT-29 cell line, established in 1964 (34), has retained specific binding sites for VIP (12), EGF, and insulin (35) as in normal intestinal epithelial cells, and it possesses D1 and D2 dopamine receptors (36). HT-29 cells have also the same number of chromosomes and the same Blood Group A marker, as previously described (12). This cell line was able to differentiate into enterocyte-like cells and mucous-like cells in culture and to induce tumors in the nude mouse (37, 38). Cells were cultured for a wk in DMEM supplemented with 10% fetal bovine serum (Gibco). HT-29 cells in culture flasks were equilibrated with an atmosphere of O2/N2/CO2 at 20, 70, and 100% and were studied in their undifferentiated stages between passages 172 and 190. Cells were cultured for a wk in 75-cm2 flasks until they reached 70 to 80% confluence. They were then washed in Ca2+-, Mg2+-free phosphate buffer and harvested after the addition of 0.025% trypsin (Millipore; 190 units/ mg) containing 25 mg of EDTA per 100 ml of Gibco solution. For experimental purposes, 3 × 10^5 cells were seeded in 9.6-cm2 Falcon plastic tissue culture dishes, and the medium was changed on Days 3, 4, and 5. All experiments (except when indicated) were performed on adherent cell layers. Each experiment was performed at least twice, using duplicate dishes.

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1 Supported in part by the Association pour la Recherche sur le Cancer.
2 To whom requests for reprints should be addressed.
3 The abbreviations used are: VIP, vasoactive intestinal peptide; 125I-VIP, VIP monoiodinated on tyrosine residue; cAMP, adenosine 3’5’-cyclic monophosphate; HPLC, high-pressure liquid chromatography; DMEM, Dulbecco’s modified Eagle’s medium containing 25 mm glucose; BSA, bovine serum albumin; IBMX, 3-isobutyl-1-methylxanthine; EGF, epidermal growth factor; EC50, concentration effecting half-maximal stimulation.
DESENSITIZATION TO VIP IN HUMAN COLONIC CARCINOMA CELLS

Desensitization Experiments. On Day 6, the culture medium was withdrawn, and confluent cells were carefully washed with 2 ml of DMEM, pH 7.4. Unless indicated otherwise, HT-29 cells were desensitized in the presence of $10^{-8}$ M VIP by incubations at 37°C in DMEM buffer supplemented with 2% (w/v) bovine serum albumin (Fraction V; Pentex). Control cells were incubated under the same conditions in the absence of VIP. Two or three dishes containing cells grown in the same conditions and submitted to the same procedure were used to check the cell viability and number. In all experiments, cells retained their morphological characteristics under microscopic examination and excluded trypan blue (>90%). Cell number determination was reproducible with variations less than 10%. In some experiments, cells were detached from the dishes and used in the study of VIP binding and cAMP production. However, in such cases VIP binding fell rapidly, and the production of cAMP was much less sustained at 37°C than at 15°C (data not shown). This is due to the rapid inactivation of both VIP and VIP receptors, as previously demonstrated (6). The percentage of cells that were colored by trypan blue after 3 h of incubation was much lower in cultured (10 ± 1.6%) than in isolated cells (22 ± 2.4%).

Measurement of VIP Receptor Activity after VIP-induced Desensitization. After the desensitization step performed at 37°C, the temperature was lowered to minimize the membrane receptor traffic during the washing steps and assays of VIP receptor activity, as indicated in the figure legends. Monolayers were washed 4 times with DMEM to remove VIP. The overall procedure of washing lasted 10 min. The assay procedure for VIP receptor activity, i.e., cAMP production and $^{125}$I-VIP binding, was performed in control and desensitized cells incubated in 1 ml of DMEM supplemented with 2% BSA (pH 7.4). It was verified that, at 15°C, $^{125}$I-VIP binding was maximal and remained constant during the 60- to 90-min incubation, allowing the measurement of receptor occupancy in conditions of apparent equilibrium (data not shown). cAMP was measured by radioimmunoassay with a succlinylation step (39) using the specific antibody 301-8, as previously described (40). At the end of incubation, 1 ml of culture medium was removed and added to 100 $\mu$l of 11 N HClO$_4$ for the determination of extracellular cAMP concentrations. Intracellular cAMP was extracted from the remaining cell monolayers by adding successively 100 $\mu$l of 11 N HClO$_4$ and 1 ml of incubation buffer. Binding assay was initiated by the addition of 0.15 to 1 x $10^{-10}$ M $^{125}$I-VIP to HT-29 cells in Petri dishes. Unlabeled VIP ($10^{-4}$ M) was added simultaneously with the label in parallel dishes to measure the nonspecific binding, which accounts for 15% or less of the total binding. At the end of binding assay, the monolayers were rapidly washed 4 times with chilled phosphate-buffered saline (pH 7.4) containing 2% BSA for the first and second washes in order to remove the unbound ligand. All results are expressed as specific binding (total minus nonspecific binding).

Determination of Cell Surface and Intracellular $^{125}$I-VIP. After incubation in the presence of radiolabeled VIP, surface-bound $^{125}$I-VIP was dissociated from the cells without removing the internalized peptide. Cell layers were treated for 6 min at 4°C with 0.7 ml of acetic acid (0.2 M, pH 2.5) containing 5.0 M NaCl, as previously described for the removal of $^{125}$I-EGF bound to ST3 cells (41). Petri dishes were then rinsed with 300 $\mu$l of the same solution, and the remaining cell-associated radioactivity was collected at room temperature after incubating the cells with two successive additions of 0.5 and 1 ml of 1 M NaOH.

RESULTS

Characteristics of VIP Receptor Activity in HT-29 Cells. We have determined the effects of time and temperature on VIP receptor activity using a saturating concentration of VIP ($10^{-8}$ M) on HT-29 cells attached to their Petri dishes (Fig. 1). At 37°C, the kinetics of VIP-induced cAMP accumulation clearly demonstrated the rapid development of the refractoriness to VIP (Fig. 1A). In the absence of phosphodiesterase inhibitor, VIP caused a rapid increase in intracellular cAMP (about 150-fold stimulation over basal levels within 20 min), followed by a progressive decline (Fig. 1A, O). The presence of the phosphodiesterase inhibitor IBMX (0.2 mM) potentiated VIP-induced intracellular cAMP accumulation. However, VIP does not accumulate after 10 min, indicating that the development of refractoriness is not suppressed by IBMX (Fig. 1A, A). Extracellular cAMP levels (Fig. 1A, O, A) increased at a much slower rate than the intracellular concentrations, and they were much lower in the absence (O) than in the presence of IBMX (A). In the absence of IBMX, almost all of the cAMP generated at 20 min was contained within the cells. At 15°C, intracellular cAMP levels were constant between 30 and 60 min, corresponding to 80-fold basal levels (Fig. 1B, O). Lowering the temperature of the assay to 10°C (Fig. 1B, A, A) decreased VIP-induced cAMP accumulation measured at 60 min in the extracellular and intracellular compartments to 3 ± 0.2 and 105 ± 9 pmol of cAMP per 10$^6$ cells, respectively. Subsequent results are presented as the sum of intra- and extracellular cAMP. Extracellular cAMP concentration measured at 60 min accounted for 8.3 and 2.7% of the total cAMP produced at 15 and 10°C, respectively.

Desensitization of HT-29 Cells to VIP. Desensitization was induced by incubating the attached HT-29 cells with $10^{-8}$ M VIP at 37°C for different times varying from 0 to 180 min (Fig. 2). After removal of the desensitizing dose, the temperature was lowered by serial washes, and the same dose of VIP was applied to the cells in order to test the activity of the VIP receptor. We have measured cAMP generation in response to VIP in nonpretreated cells, control cells (Fig. 2, O), and the residual cAMP levels in cells which were preincubated in the presence of the desensitizing dose of VIP and subsequently incubated in the absence of peptide, VIP-pretreated control cells (Fig. 2, A). This residual cAMP was generated during the VIP desensitization step and by the remaining activity of the VIP receptor during and after the washing step. The temperatures in the washing step and incubation were lower in Fig. 2B than in Fig. 2A (see legend). In both experiments, the cAMP values are much lower in VIP-pretreated cells in the presence of VIP in the challenge incubation (VIP desensitized) than in controls. Desensitization to VIP was dependent upon how long the cells were preexposed to VIP (Fig. 2, O). The loss of sensitivity to VIP was slow and maximal only after 1 h in experiments shown in Fig. 2A, while desensitization was rapid (half-time, <3 min) in experiments shown in Fig. 2B, suggesting a critical role of the temperature during the washing step and incubation in the evaluation of cell refractoriness to VIP. The desensitization of HT-29 cells to VIP is not suppressed in the presence of the...
DESENSITIZATION TO VIP IN HUMAN COLONIC CARCINOMA CELLS

Fig. 2. Time-course of desensitization to VIP. Control cells (○), VIP-pretreated cells (△), and cells desensitized with VIP (■) were compared for their cAMP generation capacity under basal conditions (○) or in the presence of 10⁻⁸ m VIP (△, ■). In a first step, 10⁻⁴ m VIP was added to desensitize the cells at 37°C, as indicated in "Materials and Methods." After exposure to VIP for the time indicated, VIP was removed with the medium. In a second step, cell monolayers were washed and reincubated in the absence of IBMX to measure cAMP production. Two different conditions were used in the second step to lower the temperature after removal of VIP. In A, cells were washed at 20°C, and cAMP was assayed after 30-min incubation at 15°C. In B, cells were washed at 10°C, and cAMP was assayed after 60-min incubation at 10°C. Each point represents total cAMP generation (intra- and extracellular). Intracellular cAMP levels accounted for more than 90% of total cAMP generated for each experiment. Bars, SE.

Fig. 3. Dose-response curves for VIP-induced stimulation of cAMP production in control and desensitized HT-29 cells. Cells were preincubated at 37°C for 180 min in two different sets of 75-cm² culture flasks containing: (a) only the medium (control cells, ○); or (b) 10⁻⁸ m VIP (desensitized cells, ■). HT-29 cells were then harvested by incubation at 37°C for 5 min in Ca²⁺- and Mg²⁺-free phosphate-buffered saline (0.13 M, pH 7.2) containing EDTA (1/5000, v/v). Isolated cells were washed 4 times at 20°C and incubated for 30 min at 15°C in the presence of different concentrations of VIP, as indicated in the abscissa. Total cAMP levels were measured as described in "Materials and Methods." Inset, magnification of the representation at low VIP concentrations (10⁻¹⁰ to 3 x 10⁻⁹ M). Bars, SE.

phosphodiesterase inhibitor IMBX. cAMP generation in response to 10⁻⁸ M VIP increased from 4.2 ± 1.7 to 762 ± 39 pmol of cAMP in control HT-29 cells incubated for 30 min at 15°C in the presence of 0.2 mm IBMX. Under the same experimental conditions, the cAMP values were not significantly different in VIP-pretreated cells incubated in the absence (residual cAMP levels) or presence of 10⁻⁸ M VIP: 198 ± 30 and 277 ± 38 pmol/10⁶ cells, respectively.

Concentration-Response to VIP in Control and Desensitized Cells. In the experiments shown in Fig. 3, adherent cell monolayers in 75-cm² culture flasks were desensitized for 180 min at 37°C in the presence of 10⁻⁸ M VIP. Then, HT-29 cells were harvested, washed, and incubated with increasing doses of VIP. In controls, maximal cAMP responses occurred at 10⁻⁸ M VIP. A considerable decrease in VIP-induced cAMP generation was observed at concentrations above 10⁻⁸ M VIP in desensitized cells. The persistence of the cAMP generation in response to 10⁻⁸ M VIP accounted for 30% of the maximal response measured in control HT-29 cells. This activation was partly due to the desensitization process that occurred during the harvesting procedure from the culture flasks (5-min incubation at 37°C with EDTA). cAMP was also slightly accumulated in HT-29 cells during the desensitization step, from 1.8 ± 0.4 to 8.2 ± 1.9 pmol/10⁶ cells in control and VIP-pretreated cells, respectively.

Concentration-Dependence and Specificity of VIP Receptor Desensitization. The sensitivity and the chemical specificity of the desensitization process were investigated by comparing the effect of increasing doses of VIP and its native analogue secretin. Cells were then challenged with either 10⁻⁸ M VIP (Fig. 4A) or 3 x 10⁻⁶ M secretin (Fig. 4B). As shown in A, the concentration of peptides causing half-maximal desensitization was 4 x 10⁻⁸ M VIP and 6 x 10⁻⁷ M secretin. Thus, VIP receptor desensitization induced by VIP and secretin in HT-29 cells was observed in a range of concentrations comparable to those required to induce the activation of the VIP receptor by these peptides. Effective concentrations are about 100-fold higher for secretin than for VIP, according to the specificity of the VIP receptor in HT-29 cells. In Fig. 4B, secretin-induced cAMP generation was measured in control, nonpretreated cells (Experiment 1), in cells desensitized to 10⁻⁸ M VIP (Experiment 2), or 3 x 10⁻⁶ M secretin (Experiment 3). The activation of the VIP receptor by high doses of secretin (3 x 10⁻⁶ M) is nearly suppressed after desensitization induced by a 300 times lower concentration of VIP resulting in a 90% loss in cAMP stimulation (Experiment 2).

Effect of Forskolin on HT-29 Cells Desensitized to VIP. We have investigated the possibility that the intracellular depletion of the active adenylate cyclase might account for the observed decrease of cAMP generation in VIP-desensitized HT-29 cells. Forskolin alone (3 x 10⁻⁶ M) significantly increased cAMP levels above basal (P < 0.001), and 3 x 10⁻⁴ M forskolin induced a 300-fold stimulation (Fig. 5A, ○, control). The same dose-response curve was observed with forskolin in VIP-pretreated HT-29 cells, indicating that the desensitization process was not related to a loss of the adenylate cyclase pools. The mechanism involved in VIP receptor desensitization was therefore studied.
Fig. 5. Effect of forskolin alone or in combination with VIP on cAMP generation in control and VIP-desensitized HT-29 cells. In a first step, cells were preincubated in 9.6-cm² Petri dishes at 37°C for 3 h, in the presence of 10⁻⁸ M VIP (VIP pretreated in A or desensitized in B), or in the absence of this peptide (controls in A or B). In a second step, cells were washed at 10°C, and after 1-h incubation at 10°C, the cAMP generation induced by forskolin alone (A) or in combination with 10⁻⁸ M VIP (B) was measured in control (O), VIP-pretreated or desensitized cells ( ). In the absence of forskolin (0 on the abscissa), comparative values of cAMP, either residual for VIP-pretreated cells (•) or basal ( ) and VIP stimulated ( ) for control cells, are shown. Bars, SE.

Fig. 6. VIP binding capacity in control and HT-29 cells desensitized to VIP. Cells were either desensitized at 37°C for 3 h in the presence of 10⁻⁸ M VIP ( ) or incubated as controls without VIP (O). In a second step, HT-29 cells were washed at 20°C and incubated for 60 min at 15°C in the presence of 1.5 x 10⁻¹¹ M ¹²⁵I-VIP alone or mixed with increasing doses of unlabeled VIP at the concentrations indicated on the abscissa. The VIP binding capacity in HT-29 cells is calculated and expressed on a log/log plot by the number of molecules bound per cell for each concentration of VIP. Bars, SE.

Fig. 7. Kinetics of the compartmental partition of ¹²⁵I-VIP in HT-29 cells. Cell layers in 9.6-cm² Petri dishes were incubated at 37°C (left), 15 or 10°C (right) in the presence of 4 x 10⁻¹¹ M ¹²⁵I-VIP. At the time indicated, the medium containing ¹²⁵I-VIP was removed, and cells were washed with iced-cold buffer. The cell surface-bound ¹²⁵I-VIP (acid soluble, •) was dissociated from the internalized ¹²⁵I-VIP (acid insoluble, O) as described in “Materials and Methods.” Total ¹²⁵I-VIP bound is the sum of acid-soluble and -insoluble ¹²⁵I-VIP ( ). In insets, cell surface-bound ( , A) and internalized ¹²⁵I-VIP (O, Δ) were expressed in percentage of total ¹²⁵I-VIP bound, at 37°C (left), 15°C (circles, right), or 10°C (triangles, right). Bars, SE.

Desensitization to VIP (Fig. SB, •) precluded the potentiating effect of VIP on forskolin-induced cAMP generation since the EC₅₀ of forskolin was elevated from 2 x 10⁻⁷ M to 2 x 10⁻⁶ M in control and desensitized cells, respectively.

Characteristics of ¹²⁵I-VIP Binding in Control and Desensitized HT-29 Cells. At 37°C, VIP treatment caused a considerable decrease in the density of VIP binding sites per HT-29 cell, from 20,000 ± 2,000 to 3,000 ± 500 sites in control and desensitized cells, respectively (Fig. 6). The apparent Kᵦ of binding did not change after desensitization. The decrease in the total number of ¹²⁵I-VIP binding sites without changes in the binding affinity accounts for the corresponding changes in efficacy of VIP on cAMP production in desensitized cells (see Fig. 3). The loss of ¹²⁵I-VIP binding sites in desensitized HT-29 cells has been further investigated after preincubation for different times (0 to 180 min) with a saturating dose of VIP (10⁻⁸ M). The decrease in ¹²⁵I-VIP binding was observed as early as 3 min after the addition of VIP. After 5-, 10-, 20-, 60-, and 180-min exposure, ¹²⁵I-VIP bound to desensitized cells does not exceed 15% of the maximal binding measured in controls (data not shown).

Compartmental Partition of VIP during the Desensitization Step. To determine whether the decrease in VIP binding capacity in VIP-desensitized HT-29 cells (see Fig. 6) was due to the occupancy of cell surface sites or to a VIP-induced internalization of VIP receptors, the interaction of ¹²⁵I-VIP with HT-29 cells was analyzed as a function of time and in relation to the cellular partition of ¹²⁵I-VIP either bound at the cell surface or internalized. The kinetics of ¹²⁵I-VIP processing was followed in Fig. 7 at the temperature chosen to obtain the desensitization (37°C) and at those (15 or 10°C) used for the binding assay of VIP. At 37°C (Fig. 7, left), maximal ¹²⁵I-VIP binding occurs at 40 min and is followed by a progressive decline. The density of cell surface-bound ¹²⁵I-VIP (acid soluble) exhibits a small peak at 10 to 20 min and decreases progressively afterwards. In contrast, internalized ¹²⁵I-VIP (acid resistant) considerably increases up to 80 min. At 15°C (Fig. 7, right), maximal ¹²⁵I-VIP binding occurs after 60 min, is followed by an apparent equilibrium, and is mainly represented by the occupancy of the ¹²⁵I-
At this temperature, the rate of internalization is much slower since only 30% of the total bound 125I-VIP was internalized at 90 min. When the data are represented as the percentage of total 125I-VIP bound (Fig. 7, insets), it appears that the proportion of sites that are acid soluble and resistant considerably varies with temperature and time. At 37°C, the rate of 125I-VIP internalization is very rapid. About 50% of the radioactivity was shifted from the cell surface to the intracellular domain within 5 min (Fig. 7, left inset). At 15°C, the internalization rate does not exceed 0.5% per min of total 125I-VIP bound during 60-min incubation and decreases thereafter (Fig. 7, right inset). At both temperatures, the prevalence of 125I-VIP binding sites at the cell surface (acid soluble) during the first minutes after addition of 10^{-9} M VIP (50% at 37°C and about 100% at 15°C) suggests that in a first step, VIP is bound to the cell surface and internalized in a second step. The rate of this internalization is very rapid at 37°C and much slower at 15°C. At 10°C (Fig. 7, right inset, A and D), the binding sites are almost exclusively concentrated at the cell surface after 60- and 90-min incubation, indicating that the internalization of VIP is practically suppressed at this temperature.

The rate of binding at 37°C is also dependent on the concentration of VIP in the external medium (Fig. 8). In the presence of 10^{-8} M VIP, i.e., an excess of ligand over the number of VIP binding sites present in this model, the rate of receptor occupancy was rapid since VIP receptors were saturated within 10 min (Fig. 8, middle). Nearly 90% of VIP was internalized in these conditions (Fig. 8, middle inset). This explains that HT-29 cells are completely desensitized in a few minutes after exposure to 10^{-8} M VIP at 37°C, as shown in Fig. 2B. The loss in 125I-VIP binding sites observed in desensitized cells shortly after VIP exposure is therefore due to the processing of VIP together with its receptor from the cell surface to intracellular domains. The rate of binding is slower in the presence of 10^{-10} M VIP (Fig. 8). At 10^{-10} M VIP nearly saturates the capacity of the VIP binding sites after a 10- to 90-min incubation at 37°C (Fig. 8, right), suggesting that the saturation is obtained at about the same concentration of VIP at 37 and 15°C, when the apparent equilibrium of binding is reached (see also Fig. 6). At 90 min, the fraction of internalized VIP (Fig. 8, right inset) was the same at the three concentrations tested, indicating that the internalization of receptor-bound ligand is independent of the degree of receptor saturation achieved upon conditions of apparent equilibrium. At 3 and 10 min, the fraction of internalized VIP was dependent on VIP concentration, being lower at 10^{-10} M VIP (Fig. 8, left and middle insets). The prevalence of VIP bound at the cell surface versus internalized VIP before the apparent equilibrium suggests that, during the processing of VIP into the cells, the rate of binding is a limiting step of the internalization process which could be accelerated by increasing the concentration of ligand.

Recovery of VIP Receptor Activity. Reversal of desensitization was examined at 37°C in two different experimental conditions. After the desensitization step, VIP is removed, and cells are incubated at different temperatures in a VIP-free medium (second step or recovery step). In a third step, VIP-induced cAMP generation (Fig. 9A) and 125I-VIP binding capacity were measured in HT-29 cells (Fig. 9B). A rapid recovery of VIP-induced cAMP stimulation (33%) was observed at 37°C after 10-min incubation (Fig. 9A, O). The reversal of the desensitization process was partial, even after a 180-min incubation: VIP receptor activity was only 54% of that observed in control HT-29 cells. At 37°C, the reappearance of 125I-VIP binding sites during the recovery step (Fig. 9B, O) was also rapid (50% at 10 min) and partial (70%) between 20 and 180 min. When the temperature of the washing procedure and incubation, following the desensitization step at 37°C, was lowered and maintained at 10°C (Fig. 9, O), the magnitude of the recovery was the same, but cAMP accumulation (Fig. 9A) and 125I-VIP binding (Fig. 9B) were slower to equilibrate than in the experiments described above. Lowering the temperature of the recovery step to 20°C resulted in decelerating further the recovery of VIP-induced cAMP generation (Fig. 9A) and 125I-VIP binding capacity (Fig. 9B). No reversal of desensitization in terms of...
cAMP stimulation and 125I-VIP binding was observed when the temperature of the recovery step was 10°C. These experiments indicate that the resensitization of the HT-29 cells to VIP is temporally related to the reappearance of VIP binding sites. After removal of the ligand, functional VIP receptors are directed from intracellular domains to the cell surface according to a temperature-dependent process. Recovery of VIP receptor activity above 10°C further explains the persistence of a slight cAMP stimulation after desensitization in the conditions of the experiments shown in Fig. 3 and, to a lesser extent, in Fig. 24. In order to know if the recovery process was related to VIP receptor recycling or synthesis, we have compared the recovery of the 125I-VIP binding sites in control and desensitized cells exposed to cycloheximide, an inhibitor of protein synthesis. No significant differences were observed between those two groups (Table 1), indicating that the reappearance of functional 125I-VIP binding sites was not related to the de novo protein biosynthesis.

**DISCUSSION**

Desensitization appears to be a general process modulating the cellular response to hormones and neurotransmitters involved in the control of biological functions (reviewed in Ref. 42). Detailed studies on the interaction between VIP and the HT-29 colonic carcinoma cell line in culture presented here demonstrate for the first time that the desensitization to this neuropeptide was mediated by the VIP receptor. (a) Desensitization occurs after previous exposure to the homologous peptide and is a function of time and VIP concentration. (b) The concentration dependence of the desensitization induced by VIP and secretin corresponds to the pharmacological specificity of the VIP binding sites, the EC50 of secretin being at least 100-fold higher. (c) The loss in 125I-VIP binding sites accounts for the altered cAMP responses, as evidenced by a similar loss in the extent of stimulation by VIP, without modification of its EC50. (d) There is also a temporal relationship between the VIP-induced cAMP desensitization and the disappearance of the VIP receptor from the cell surface. Our results indicate that the receptor-mediated desensitization to VIP is associated with a transformation of the complex between the receptor bound VIP and the adenylate cyclase system. Indeed, as previously shown (5) stimulation of the adenylate cyclase by VIP involved at least three components: (a) the receptor binding proteins which bind the natural ligand or its agonists triggering the biological response; (b) the stimulatory guanyl protein which binds GTP; and (c) the catalytic moiety of the adenylate cyclase. Analysis of the cAMP response to forskolin indicated that desensitization to VIP did not modify the effect of forskolin alone. Since this ubiquitous adenylate cyclase activator has been shown to interact with the catalytic site of the cyclase, VIP receptor desensitization was therefore not associated with a loss in the adenylate cyclase pools. Desensitization, however, results in a considerable decrease in the potentiation between VIP and forskolin (see Fig. 5B). Recent data also indicated that forskolin is likely to potentiate the action of ligands by stabilizing the complex between the stimulatory guanine nucleotide-binding subunit, and the catalytic moiety of adenylate cyclase (43).

According to this model, it might be hypothesized here that the functional coupling between the VIP receptor and this stabilized complex is reduced upon desensitization to VIP. Furthermore, the loss in VIP binding capacity after desensitization is associated with a concomitant accumulation of the intracellular receptor-bound VIP. The translocation of the receptor-bound VIP inside the cells is a stoichiometric and time-dependent process which, at the VIP concentration used to desensitize cells (10^-8 M), accounts for as much as 90% of internalized VIP after 10 min at 37°C (see Fig. 8). In that respect, desensitization to VIP appears closely linked to the uptake of the bound ligand, a process named “receptor-mediated endocytosis” (44, 45; and reviewed in Ref. 46). Due to the paucity of data on VIP receptor and ligand internalization, little is known about the compartments in which this complex is present after internalization. Biochemical characterization by HPLC of internalized VIP indicated that, within 10 and 120 min of incubation, nearly 70% of the ligand remained intact (30), while the peptide progressively released in the medium was degraded (29, 30). Ultrastructural localization of radiolabeled VIP during the internalization process indicates that at 10 min, up to 60% of the internalized peptide remains located in clear vesicles inside the cells (47). Moreover, the surrounding membrane of these vesicles accounts for most of this labeling (87%), suggesting that the majority of the peptide is still attached to its receptor (47).

In corroboration with the above observations, the data presented here indicate that VIP receptor-mediated endocytosis can now be listed among the earliest events following the binding of VIP to its cell surface receptors in human colonic carcinoma cells. Upon VIP removal, the VIP binding sites quickly accumulate again at the cell surface at 37°C and are reinserted in the plasma membrane in such a way that they can be reactivated (see Fig. 9). Therefore, a set of functional VIP binding sites has not been masked during endocytosis and retains its ability to be coupled and to activate again the subunits of the cyclase. Since the recovery of binding sites is not dependent on protein synthesis (see Table 1), the two most likely possibilities are receptor recycling or membrane insertion of intracellular receptors from an endogenous pool (46). The partial recovery of VIP receptor activity observed here suggests that the internalized receptors might follow two different processes, one related to a rapid recycling as described above, the other related to receptor degradation or inactivation. The latter mechanism has also been termed “down-regulation” (48). The existence of different pathways in the receptor-mediated endocytosis of VIP opens the field to study under which different

**Table 1** Effect of cycloheximide on the recovery of 125I-VIP binding capacity in HT-29 cells desensitized to VIP

<table>
<thead>
<tr>
<th>Experimental design</th>
<th>Cycloheximide (µg/ml)</th>
<th>Specific 125I-VIP binding/Petri dish (% of total radioactivity)</th>
<th>Specific 125I-VIP binding (% of controls)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>0</td>
<td>6.6 ± 0.6*</td>
<td>100</td>
</tr>
<tr>
<td>Controls</td>
<td>1</td>
<td>7.5 ± 0.7</td>
<td>100</td>
</tr>
<tr>
<td>Recovering cells</td>
<td>0</td>
<td>3.8 ± 0.2</td>
<td>58</td>
</tr>
<tr>
<td>Recovering cells</td>
<td>1</td>
<td>4.4 ± 0.4</td>
<td>58</td>
</tr>
</tbody>
</table>

* Mean ± SE.
circumstances of carcinoma and normal intestinal epithelial cells either recycle or inactivate the internalized receptor-bound ligand.

The physiological implication of desensitization to VIP is still unknown. The desensitization in gut carcinoma epithelial cells fits well with the transient activity of VIP in stimulating phosphorylase a and mobilizing glycogen, as previously demonstrated in our laboratory (25). The biological action of VIP was also found to be transient for the stimulation of glycogen phosphorylase in liver cells (49) or excitatory action in the brain (50). A population of mesenteric node T which had been incubated in the presence of VIP showed a reduced ability to respond to another interaction with this peptide (51). In a variety of tissues, the existence of a desensitization process has been proposed and deduced from the kinetics of VIP-induced cAMP production in intact cells. These systems included the pancreatic acinar cells (52), intestinal cells isolated from rat neonates (53), enterocyte-like HT-29 cells (54), HGT-1 human gastric cancerous cells (55), GH3 rat pituitary cells (18), and rat hippocampal slices (56). In other models and experimental conditions, the process was slow, occurring within hours and days in neonatal mouse calvaria (57), osteosarcoma cells (20), or HGT-1 cells in culture (14). These events could be referred to as long-term desensitization. In the experiments described here, VIP receptor desensitization was evaluated after washing the cells at 10°C, making it possible to detect that desensitization occurs in a few minutes (Fig. 2B), since the recovery and recycling of VIP receptors are then suppressed (Fig. 9). Therefore, the subsequent blockade of the recycling process by low temperature allowed us to detect the onset of endocytosis as an early event in HT-29 cells exposed to VIP. Furthermore, those results explain that, when VIP binding or VIP-induced cAMP production persists to some extent after desensitization (see Figs. 2A, 3, and 6), it is due to an incomplete blockade of the temperature-dependent receptor recycling process. Interestingly, the measured EC50 values for the VIP receptors that reappear at the cell surface were not different from those initially characterized at the cell surface. Therefore, the process of desensitization and recovery was related to the internalization and recycling of the VIP receptor rather than to functional changes resulting from the interaction with the ligand.

Finally, it appears that two different processes are involved in the desensitization of HT-29 cells to VIP. Early events are associated with the rapid endocytosis of the VIP-bound receptor, precluding any stimulation of this receptor by subsequent addition of the peptide. The magnitude of this process depends upon the concentration of the desensitizing dose of VIP and of the relative rate of receptor internalization versus recycling or cycling from intracellular pools. The long-term desensitization, on the other hand, depends on the fate of the receptors after internalization. It persists after removal of the ligand from the external medium and finally depends on the relative rate of VIP receptor destruction versus synthesis after chronic treatment by VIP. For the moment, we cannot compare the processes of VIP receptor down-regulation in carcinoma and normal intestinal epithelial cells, due to the absence of quantitative studies in normal cells. However, it is of interest to notice that VIP receptor regulation by VIP is observed in a range of concentrations of 10^{-9} to 10^{-8} M that are comparable with the release of this neurotransmitter by nerve endings at the vicinity of the intestinal epithelial cells (58, 59). Some differences are noticeable between the regulation of VIP receptor described here and that of insulin receptors described in the same model (60). The uptake of ^{125}I-insulin is slower, and the decrease in insulin receptors was mainly observed at high insulin concentrations (10^{-4} M), indicating a tumor-associated resistance to receptor down-regulation for this hormone. The rapid and considerable desensitization to VIP presented here, together with a resistance to insulin-induced receptor down-regulation, may be of considerable importance in tumor cell biology. The growth and stimulation of DNA synthesis in carcinoma colonie cells in culture may be induced by low doses of insulin (61), while the elevation of cAMP levels in rat intestinal cells might have an opposite effect by suppressing DNA synthesis. Therefore, the rapid desensitization to VIP reported here might be related to the biochemical events linked to the growth of cancerous cells. The evidence of rapid desensitization associated with rapid endocytosis in the experiments described here underlines the interest of the HT-29 human colonic carcinoma cells as a model for understanding the regulatory mechanisms of VIP action as a neurotransmitter. Furthermore, using this model it is now possible to study pharmacological and natural agents which can modify the rate of internalization, the positioning, and the traffic of internalized VIP receptors, as well as the relative rates of their recycling and destruction, and to act through these processes on the growth of human cancerous gut epithelial cells.

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C. Boissard, J-C. Marie, G. Hejblum, et al.