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0014-4754/89/11-12/1099-04$1.50 + 0.20/0


## VIP receptors and control of short circuit current in the human intestinal clonal cell line Cl.19A

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*Received 13 March 1989; accepted 22 May 1989*

*Summary.* At the maximally effective concentration of 10 nM, VIP induced a marked (12.5-fold stimulation above basal), and sustained increase in short circuit current in the human intestinal epithelial cell line Cl.19A grown on permeable filters and placed in Ussing chambers. Half-maximal increase of Isc was observed for 0.1 nM VIP. This was well correlated with the VIP-stimulated adenylate cyclase activity ($ED_{50}$: 0.07 nM). Binding studies using $^{125}$I-VIP indicated that Cl.19A cells express a peptide-specific VIP receptor with a dissociation constant of 0.07 nM. Covalent labeling of receptors followed by SDS-PAGE analysis of membrane proteins resulted in the identification of a 63 000 dalton binding protein in Cl.19A cells.
*Key words.* Colonic epithelial cell line; VIP receptors; short circuit current.

The neuropeptide vasoactive intestinal peptide (VIP) plays a pivotal role in controlling cyclic AMP-dependent secretion of water and electrolytes in the intestine[1]. Several cultured cell lines derived from human colonic adenocarcinomas have been used either for documenting the mechanisms whereby VIP induces chloride secretion, or for examining some properties of VIP receptors[2–5]. However, it is difficult to correlate the results derived from these two types of studies since they have been conducted independently on different models.

Therefore we were prompted to explore within the same intestinal cell line the functional and molecular characteristics of VIP receptors, the ability of VIP to stimulate adenylate cyclase, and the effects of VIP on short circuit current in cells grown on permeable filters and mounted in Ussing chambers. The human intestinal Cl.19A cell line[6] was chosen as it had previously been shown to have the properties of a chloride secreting epithelium[7].

### *Materials and methods*

Cell line: the Cl.19A[6] cell line is a differentiated clonal derivative of the human colonic adenocarcinoma cell line HT29[8]. The isolation as well as the culture characteristics of these cells have been described elsewhere[6]. Briefly, the Cl.19A cells have been shown to form, on reaching confluency, a typical monolayer with the apical cell surfaces separated from the basolateral surfaces by tight junctions.

The Cl.19A cells were routinely cultured in Dulbecco's modified Eagle's medium, plus 10% heat inactivated foetal bovine serum. The cells were routinely screened for mycoplasma contamination using Chen's method[9] and they were always found to be negative.

For electrical analysis of transport function, the cells were seeded at a density of 800,000 cells per $cm^2$ on collagen-coated permeable filters (diameter: 13 mm; HAHY millipore filters)[10]. On day 16 of culture, each filter was placed as a flat sheet in an Ussing chamber. Short circuit current was measured as previously described[2,3].

Membranes were prepared from post-confluent cells as described[10]. Adenylate cyclase activity was assayed following the previously published method[11]. The binding assay of $^{125}$I-VIP was conducted as described[11]. The incubation medium contained in a 250-µl final volume,

Amino acid sequences of VIP and related peptides

| | 1 5 10 15 20 25 30 35 40 |
|---|---|
| p VIP | H S D A V F T D N Y T R L R K Q M A V K K Y L N S I L N-NH2 |
| p Secretin | *H S D* G T *F T* S E L S *R L R* D S A R L Q R L *L* Q G L V-NH2 |
| p PHI | *H* A *D* G *V F T* S D F S *R L* L G Q L S A *K K Y L E S* L I-NH2 |
| r GRF | *H A D A* I *F* I S S *Y R R* I L G *Q* L Y A R *K* L *L* H E *I* M *N* R Q Q G E R N Q E Q R S R F N-OH |
| h GRF | Y A *D A* I *F* T N S *Y* R K V L G *Q* L S A R *K* L *L* Q D *I* M S R Q Q G E S N Q E R G A R A R L-NH2 |

The letters p, r, and h designate the porcine, rat and human species, respectively.

60 mM Hepes (pH 7.5), 2 mg/ml bovine serum albumin, 1 mg/ml bacitracin, $^{125}$I-VIP at $5 \times 10^{-11}$ M and unlabeled peptides when necessary. After a 30-min incubation at 30 °C, membrane-bound $^{125}$I-VIP was determined by centrifugation as described[11]. Results are expressed as specific binding; this is obtained by subtracting from the total bound that amount of $^{125}$I-VIP which remained bound in the presence of 1 μM native VIP. Cross-linking of $^{125}$I-VIP to receptors was obtained as described[12] using dithiobis (succinimidylpropionate) as a cross-linker. Subsequent analysis of membrane proteins was performed by SDS-PAGE according to the procedure of Laemmli[13]. Samples were analysed under non-reducing conditions on a 5–15% polyacrylamide slab gel with a 3% stacking gel as described in detail[12]. Membrane proteins were determined according to Bradford[14], using bovine serum albumin as standard.

Reagents: purified porcine VIP was provided by Dr. V. Mutt (Karolinska Institute, Stockholm, Sweden), synthetic VIP 2–28 by Prof. S. St Pierre (University of Sherbrooke, Quebec, Canada), synthetic peptide histidine isoleucineamide (PHI) and secretin by Prof. L. Moroder (Max Planck Institute, Martinsried, Germany) and synthetic human(h) growth hormone releasing factor (GRF)-44-$NH_2$ and rat(r)GRF by Prof. Guillemin and N. Ling (The Salk Institute for Biological Studies, San Diego, CA, USA). The amino acid sequences of VIP and related peptides are shown in the table. $^{125}$I-VIP was prepared by the chloramine T method at a specific activity of 250 Ci/g as described[15]. It displayed the same activity as native VIP in stimulating cyclic AMP production in the HT-29 cell line[16]. Chemicals and unstained protein markers for SDS-gel electrophoresis were purchased from Bethesda Research Laboratories, Guthersburg, MD and dithiobis (succinimidylpropionate) from Pierce, Rockford, IL. Bumetanide was obtained from Laboratoires LEO (Paris, France) and dissolved in ethanol as a $10^{-2}$ M stock solution. Other chemicals, of the highest purity commercially available, were obtained from Sigma, St Louis, MO.

## *Results*

The addition of $10^{-8}$ M VIP to the serosal side of Cl.19A cells grown on permeable filters and placed in Ussing chambers elicited a 12.5-fold stimulation of short-circuit current above the basal level (fig. 1). The VIP-stimulated Isc values reached a plateau after 10 min, which lasted

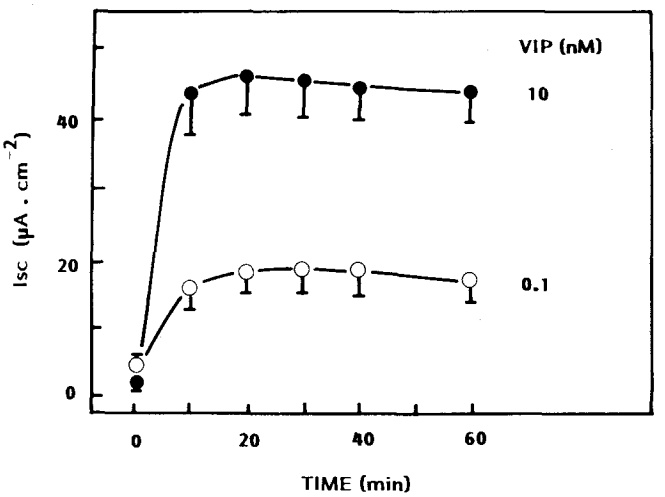


Figure 1. Electrical response of filter-grown cells to VIP. Filters were placed in Ussing chambers. After a 20-min equilibration period, VIP was added to the serosal side. For each concentration short-circuit current (Isc) values are means ± SE of 4 filters.

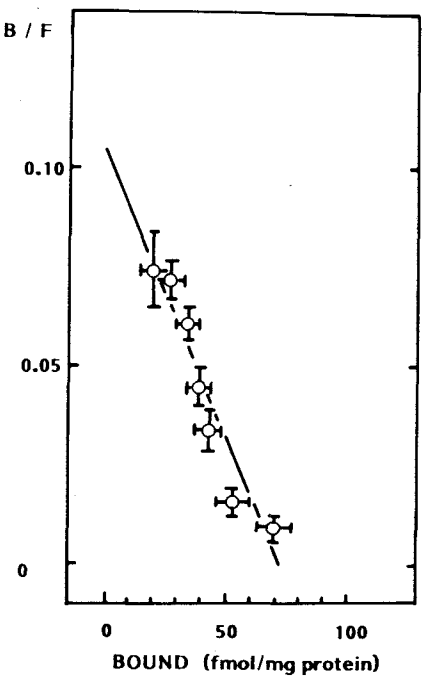


Figure 2. Scatchard plot of $^{125}$I-VIP binding to Cl.19A cell membranes. Conditions were as described in 'Materials and methods'. Values are means ± SEM from 4 separate experiments.

until at least 60 min. The addition of a higher concentration of the peptide failed to produce any further stimulation (not shown). Half maximal stimulation of Isc was observed for 0.1 nM VIP (fig. 1). When serosal bumetamide ($10^{-4}$ M) was added at the plateau of VIP-stimulated Isc, a rapid decrease of Isc was observed; it reached about one third of its initial value after 20 min (not shown).

Equilibrium binding studies of $^{125}$I-VIP to membranes prepared from postconfluent Cl.19A cells gave linear Scatchard plots which were compatible with the presence of one population of non-interacting receptors in this cell line (fig. 2). The quantitative characteristics of VIP receptors as determined from 4 independent experiments can be summarized as follows; the binding capacity was $71 \pm 7$ fmol/mg of protein with a $K_d$ of $0.07 \pm 0.01$ nM. When the peptide specificity of receptors was examined using several naturally-occurring VIP-related peptides (fig. 3), the following order of potency in inhibiting $^{125}$I-VIP binding to membranes was observed: VIP > rGRF > PHI = hGRF > secretin.

In order to characterize VIP receptors at the molecular level, $^{125}$I-VIP was covalently linked to receptor sites in membranes from Cl.19A cells; then SDS-PAGE analysis of membrane proteins revealed a major band of $M_r$ 66,000 whose labeling was completely abolished when 0.3 µM unlabeled VIP was added together with labeled VIP (fig. 4). Assuming one molecule of $^{125}$I-VIP ($M_r$ 3300) is bound per molecule of protein, a protein of $M_r$ 63,000 was identified as the VIP binding site.

It is well known that VIP exerts its effects by stimulating the production of cAMP in its target cells [1]. Conversely dbcAMP was found to increase Isc values in Cl.19A cells [7]. Thus it was of interest to investigate the effects of VIP on adenylate-cyclase activity in this cell line.

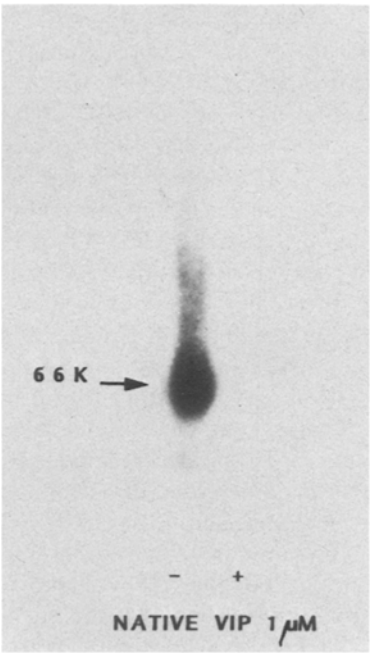


Figure 4. Pattern of covalent labeling of Cl.19A cell membrane proteins by $^{125}$I-VIP determined by sodium dodecylsulfate-polyacrylamide gel electrophoresis. Membranes were incubated with $^{125}$-VIP in the absence or presence of 1 µM unlabeled VIP, then treated with 1 mM crosslinking agent as indicatd in 'Material and methods'.

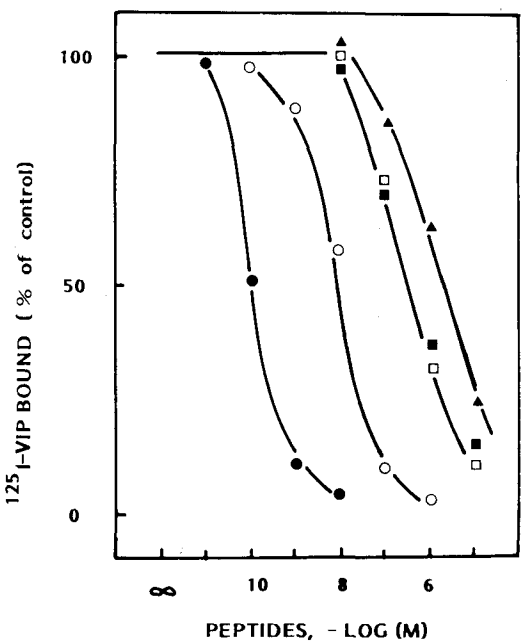


Figure 3. Competitive inhibition of specific $^{125}$I-VIP binding to membranes from Cl.19A cells by VIP and VIP-related peptides. Conditions were as described in 'Materials and methods'. Results are expressed as the percentage of radioactivity specifically bound in the absence of unlabeled peptides. Membranes were incubated with the indicated concentrations of VIP (●), rGRF (○), hGRF (□), PHI (■), or secretin (▲). Each point is the mean of 3 determinations. For clarity, standard errors are not indicated; they are always below 12% of mean values.

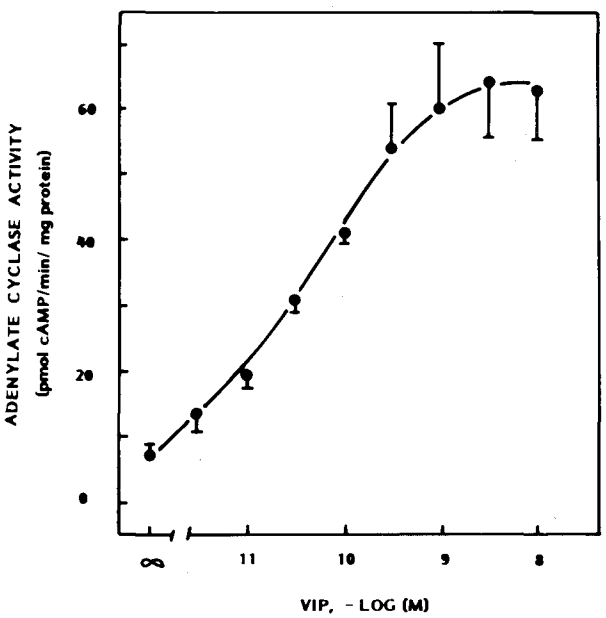


Figure 5. Adenylate cyclase activity in Cl. 19A cell menbranes in response to increasing concentrations of VIP. Conditions were as described in 'Material and methods'. Values are means ± SEM from 4 determinations.

VIP stimulated enzyme activity in membranes prepared from Cl.19A cells in the range of concentration of $10^{-11}$–$10^{-8}$ M with an $ED_{50}$ of $0.07 \pm 0.02$ nM VIP (fig. 5).

*Discussion*

Our finding in the Cl.19A cell line of a strong correlation between three parameters associated with VIP receptor-mediated biological events; namely, dissociation constant of receptor, $ED_{50}$ for activation of adenylate cyclase and $ED_{50}$ for increasing short circuit-current, clearly suggests that this cell line is a valid model for unraveling the cellular mechanisms involved in an important physiological function, i.e. intestinal secretion of electrolytes. The relevance to physiology of the studies performed on the Cl.19A cell line is further supported by the fact that the dissociation constant of the VIP receptor, its ability to discriminate between several VIP-related peptides, and its molecular size in these cultured human intestinal cells are the same as those previously reported in epithelial cells isolated from normal human colon[11,12,17,18].

Previous work has led to the isolation of several differentiated clonal derivatives from the undifferentiated HT29 cells, each of these clonal lines displaying distinctive features of intestinal differentiation. The function of ionic transepithelial transport is represented by Cl.19A cells[7], and the function of mucus secretion is typified by the Cl.16E cells[6]. It is worthwhile to emphasize the identical expression of VIP receptors in the 2 above-cited clonal cell lines (this paper and Laburthe et al.[10]). This includes dissociation constant and concentration of receptors, peptide specificity, molecular weight of the VIP receptors, and coupling to the adenylate cyclase system. Therefore it may be stated that VIP receptors are expressed in 2 lineages of intestinal differentiation, ending in cells with different functions, e.g., ion transport and mucus secretion. Whereas VIP stimulates chloride secretion in Cl.19A cells, it potentiates the cholinergic stimulation of mucus secretion in Cl.16E cells[10].

*This work was supported in part by the Association Française de Lutte contre la Mucoviscidose (AFLM), by the Association pour la Recherche sur le Cancer (ARC), and the Ligue Nationale Française contre le Cancer.* Dr. Grasset's present address is: Roussel Uclaf Nutrition, Tour Roussel Hoechst, 1 Terrasse Bellini, F-92800 Puteaux.

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0014-4754/89/11-12/1102-04$1.50 + 0.20/0


## The influence of in vitro sodium and potassium ion ratio on teleost melanosome intracellular motility

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*Received 17 January 1989; accepted 14 June 1989*

*Summary.* The miniature neuroeffector system represented by the melanophore and neural elements of winter flounder (*Pseudopleuronectes americanus*) scale slips demonstrates asymmetrical sensitivity to progressive in vitro changes in the relative concentrations of $Na^+$ and $K^+$ ions. $K^+$ ions induce melanosome aggregation more readily than $Na^+$ ions evoke melanosome dispersion, reflecting the influence of $K^+$ induced depolarization on neurotransmitter release.
*Key words.* Melanophores; integumentary nerve plexus; sodium ions; potassium ions; neurotransmitter.