Molecular identification and structural requirement of vasoactive intestinal peptide (VIP) receptors in the human colon adenocarcinoma cell line, HT-29

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The human colon adenocarcinoma cell line HT-29 in culture exhibits a cyclic AMP production system highly sensitive to vasoactive intestinal peptide (VIP), making HT-29 cells a unique cultured cell system for studying the mechanism of VIP action [Laburthe, Rousset, Boissard, Chevalier, Zweibaum & Rosselin (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 2772–2775]. The quantitative characteristics of VIP receptors in HT-29 cells and their structural requirement and molecular size were studied. ^{125}I -labelled VIP bound in a time-dependent manner to HT-29 cell homogenates. At equilibrium (60 min incubation at 30 °C), unlabelled VIP in the 0.01–10 nM concentration range competed with ^{125}I -VIP for binding to cell homogenates. Scatchard analysis of binding data gave a straight line, indicating that VIP bound to a single population of sites with a K_D of 0.12 ± 0.02 nM and a capacity of 120 ± 9 fmol/mg of protein. The structural requirement of these receptors was studied with peptides structurally related to VIP, either natural or synthetic. Several peptides inhibited ^{125}I -VIP binding to HT-29 cell homogenates with the following order of potency, which is typical of the human VIP receptor: VIP ($\text{IC}_{50} = 0.1$ nM) > VIP-(2–28)-peptide ($\text{IC}_{50} = 13$ nM) > human growth hormone releasing factor ($\text{IC}_{50} = 56$ nM) > peptide histidine isoleucine amide ($\text{IC}_{50} = 80$ nM) > secretin ($\text{IC}_{50} > 10000$ nM). To characterize the molecular component(s) of the VIP receptor in HT-29 cells, ^{125}I -VIP was covalently bound to cell homogenates by using the cross-linker dithiobis(succinimidyl propionate). Sodium dodecyl sulphate/polyacrylamide-gel autoradiographic studies of affinity-labelled cell homogenates revealed two major bands, corresponding to ^{125}I -VIP-protein complexes of M_r 66000 and 16000. The labelling of the M_r -66000 component was specific, since it was abolished by native VIP, whereas that of the M_r -16000 component was not. Densitometric scanning of autoradiographs indicated that the labelling of the M_r -66000 complex was inhibited by low VIP concentrations in the 0.1–10 nM range ($\text{IC}_{50} = 0.6$ nM), but was unaffected by $1 \mu\text{M}$ -glucagon or octapeptide of cholecystokinin. It was also decreased by VIP-(2–28)-peptide with a potency 1% that of VIP. Assuming that one molecule of ^{125}I -VIP bound per molecule of protein, one protein of M_r 63000 was identified as a component of the VIP receptor in HT-29 cells.

INTRODUCTION

The vasoactive intestinal peptide (VIP) is a neuropeptide widely distributed in the organism where it regulates various peripheral and central functions (Said, 1982). VIP triggers biological responses through specific binding to cell surface receptors (Laburthe *et al.*, 1978*a*). The functional properties of VIP receptors as well as their coupling to adenylate cyclase have been described extensively, in particular in the rat intestinal epithelium (Laburthe & Dupont, 1982). As early as 1978, we reported that VIP receptors are present in cell lines derived from human colon adenocarcinoma (Laburthe *et al.*, 1978*b*) and later confirmed their wide distribution in human colonic cancerous cells (Laburthe *et al.*, 1980*a*). Owing to the dramatic sensitivity of its cyclic AMP production system to VIP, we proposed that one of these cell lines, HT-29, may be a unique cellular model in culture for studying the mechanism of VIP action (Laburthe *et al.*, 1978*b*). Indeed, HT-29 cells have been

subsequently used by us and others for various purposes such as the transfer of VIP receptors from HT-29 cells to the adenylate cyclase in another cell (Laburthe *et al.*, 1979), the study of VIP control of protein kinases (Marvaldi *et al.*, 1979) and glycogenolysis (Rousset *et al.*, 1981), the elaboration of a highly sensitive bioassay for VIP (Laburthe *et al.*, 1980*b*) and the generation of antibodies that partially antagonize the VIP-stimulated adenylate cyclase activity (Pichon *et al.*, 1983). In spite of the frequent use of HT-29 cells for studying the dynamics of VIP receptors, very little is known about their basic properties in HT-29.

The present work had three main objectives. Firstly, we have determined the quantitative characteristics of VIP binding to HT-29 cells with respect to dissociation constant and concentration of sites. Secondly, we have analysed the structural requirements of VIP receptors, using various natural or synthetic peptides structurally related to VIP. Thirdly, by using the cross-linking procedure developed to analyse the molecular properties

Abbreviations used: DTSP, dithiobis(succinimidyl propionate); VIP, vasoactive intestinal peptide; PHI, peptide histidine isoleucine amide; hGRF, human growth hormone releasing factor (somatoliberin); SDS, sodium dodecyl sulphate.

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of rat intestinal VIP receptors (Laburthe *et al.*, 1984), we have studied the HT-29 cell component(s) responsible for VIP binding. We provide evidence that in HT-29 cells VIP binds to a single population of high-affinity sites of M_r 63 000. The structural requirements and molecular size of these sites are different from those described in the rat intestine (Laburthe *et al.*, 1983, 1984), but compare well with those reported in normal human colon (Laburthe *et al.*, 1983; Couvineau & Laburthe, 1985).

EXPERIMENTAL

Materials

Porcine VIP was provided by Professor V. Mutt (Karolinska Institute, Stockholm, Sweden). ^{125}I -labelled VIP was prepared by the chloramine-T method (Hunter & Greenwood, 1962), at a specific radioactivity of 250 Ci/g, as described by Laburthe *et al.*, (1977). It displays the same activity as native VIP in stimulating cyclic AMP accumulation in the cultured cell line HT-29 (Laburthe *et al.*, 1978b). Synthetic VIP-(2-28)-peptide was synthesized by a solid-phase method (Fournier *et al.*, 1984) and generously supplied by Dr. S. St. Pierre (University of Sherbrooke, Quebec, Canada). Synthetic porcine secretin was given by Professor E. Wunch and Professor L. Moroder (Max Planck Institute, Martinsried, Germany). PHI (Tatemoto & Mutt, 1980) was kindly provided by Dr. K. Tatemoto (Karolinska Institute, Stockholm, Sweden), and GRF was provided by Dr. N. Ling (Salk Institute, San Diego, CA, U.S.A.).

Other compounds were obtained from the following sources: porcine pancreatic glucagon, Novo (Bagvaerdt, Denmark); octapeptide of cholecystokinin, Peninsula Laboratories (Belmont, CA, U.S.A.); DTSP, Pierce Chemical Co. (Rockford, IL, U.S.A.); calibrating proteins, Bethesda Research Laboratories (Gaithersburg, MD, U.S.A.); Dulbecco's modified Eagle's minimum essential medium, Eurobio, (Paris, France); fetal bovine serum, Boehringer (Mannheim, Germany); plastic flasks, Corning Glassworks (Corning, NY, U.S.A.). Other chemicals were of highest purity commercially available.

Cell culture and preparation of particulate fraction from cell homogenate

The human colon adenocarcinoma cell line HT-29 (Fogh & Trempe, 1975) was obtained from Dr. J. Fogh (Sloan Kettering Institute for Cancer Research, Rye, NY, U.S.A.). The cells, which are routinely weekly passaged, were studied at passage 180. The cells were seeded at 1×10^6 cells in 25 cm² plastic flasks in Dulbecco's modified Eagle's minimum essential medium with 10% (v/v) fetal bovine serum and incubated at 37 °C in a CO₂/air (1:9) atmosphere. The medium was changed daily. Cells were harvested on day 4-5 (in exponential growth phase) with 3 mM-EDTA in 0.13 M-phosphate-buffered saline pH 7.2. They were centrifuged (2000 g for 5 min at 4 °C) and the cell pellet was resuspended in hypo-osmotic 5 mM-Hepes buffer, pH 7.5. After 15 min in ice, the cell suspension was homogenized with 30 strokes of a Dounce homogenizer. The homogenate was centrifuged at 30000 g for 10 min. The resulting pellet was washed three times in 60 mM-Hepes buffer, pH 7.5, and kept at -80 °C until use. This membrane/particulate fraction obtained from cell homogenate will be referred

to as particulate preparation. Proteins were determined by the method of Bradford (1976), with bovine serum albumin as standard.

Binding of ^{125}I -VIP

Particulate preparation (about 100 µg/ml) was incubated at equilibrium for 60 min at 30 °C with ^{125}I -VIP (0.05 nM for binding studies or 0.3 nM for cross-linking studies) and other compounds when necessary in 60 mM-Hepes buffer, pH 7.5, containing 2% (w/v) bovine serum albumin and 0.1% (w/v) bacitracin. The reaction was stopped by transferring the incubation medium to an excess of ice-cold buffer, and homogenate-bound labelled VIP was separated by centrifugation at 30000 g for 10 min at 4 °C. For each unlabelled peptide the IC₅₀ was defined as the concentration of peptide which half-maximally inhibited the specific binding of ^{125}I -VIP.

Cross-linking conditions and SDS/polyacrylamide-gel electrophoresis of proteins

Particulate preparation containing bound ^{125}I -VIP were suspended in 1 ml of 60 mM-Hepes buffer, pH 7.5, and incubated for 15 min at 4 °C in the presence of 1 mM-DTSP as described by Laburthe *et al.* (1984). At the end of the incubation, the reaction was quenched by adding 500 µl of ice-cold 60 mM-Hepes buffer, pH 7.5, containing 60 mM-lysine. The cross-linked material was pelleted at 20000 g for 15 min, and the resulting pellets were suspended by five successive passages through a 25-gauge needle in 60 mM-Tris/HCl buffer, pH 6.8, containing 10% (v/v) glycerol, 0.001% (w/v) Bromophenol Blue and 3% (w/v) SDS. After heating at 60 °C for 30 min, the suspension was centrifuged at 40000 g for 15 min and aliquots of the supernatant were applied to a 5-20%-polyacrylamide slab gel (1.5 mm thick), in accordance with the Laemmli (1970) method with a 3%-acrylamide stacking gel. The gels were run, fixed, stained and destained as described by Laburthe *et al.*, (1984). They were dried and exposed for 3-8 days at -80 °C to a Trimax type XM film (3M) with a Trimax intensifying screen. Autoradiographs were scanned at 600 nm with a spectrophotometer connected to an Omni-Scribe recorder (Houston Instruments).

RESULTS

Specific binding of ^{125}I -VIP to particulate preparation obtained from HT-29 cell is time-dependent (Fig. 1). At 30 °C, steady-state binding is maintained until at least 120 min. Non-saturable binding determined in the presence of 0.1 µM native VIP routinely accounts for 20% of specific binding. The concentration-dependence of VIP binding at equilibrium, i.e. 60 min incubation, is determined by adding increasing concentrations of native VIP to a fixed concentration of ^{125}I -VIP (0.05 nM). Native VIP competitively inhibits ^{125}I -VIP binding to particulate preparation in the concentration range 0.01-10 nM (Fig. 2), half-maximal inhibition being obtained at 0.10 ± 0.01 nM (mean $\pm$ S.E.M. for seven experiments). Scatchard analysis gives a straight line (Fig. 3), suggesting that VIP binds to a single population of sites. From seven separate experiments, the dissociation constant, K_D , is estimated at 0.12 ± 0.02 nM (range 0.05-0.19 nM) and the concentration of binding sites at 120 ± 9 fmol/mg of

Fig. 1. Time course of ^{125}I -VIP binding to particulate preparation at 30 °C

Conditions are as described in the Experimental section. Specific binding is expressed as percentage of total radioactivity. Values are means $\pm$ S.E.M. for triplicate determinations.

Fig. 2. Competitive inhibition of specific ^{125}I -VIP binding to HT-29 cell homogenates by hGRF, PHI, secretin, VIP-(2-28)-peptide and VIP

Conditions are as described in the Experimental section. Results are expressed as the percentage of radioactivity specifically bound in the absence of unlabelled peptide. Each point is the mean of three separate experiments. For clarity, standard errors are not indicated; they are always below 10% of the mean values.

protein (range 97–150). To determine the specificity of VIP-binding sites in HT-29 cells, a variety of polypeptides were tested for their ability to inhibit ^{125}I -VIP binding. Peptides structurally unrelated to VIP, such as insulin and octapeptide of cholecystokinin, are without effect at doses as high as 1000 nM. In contrast, peptides belonging to the structural family of VIP, such as hGRF, PHI and secretin, are able to compete with ^{125}I -VIP for binding to the HT-29 cell (Fig. 2). The same holds true for the VIP (2–28) fragment, which lacks the *N*-terminal histidine. The order of potency of the different peptides in inhibiting ^{125}I -VIP binding is: VIP ($\text{IC}_{50} = 0.1 \text{ nM}$) > VIP(2–28)-peptide ($\text{IC}_{50} = 13 \text{ nM}$) > hGRF ($\text{IC}_{50} = 56 \text{ nM}$) > PHI ($\text{IC}_{50} = 80 \text{ nM}$) > secretin ($\text{IC}_{50} > 10000 \text{ nM}$). This order of potency, as well as the intrinsic potency of each peptide, are typical of the human VIP receptor, as described previously in normal colonic epithelium (Laburthe *et al.*, 1983; Couvineau *et al.*, 1984).

Fig. 3. Scatchard analysis of competitive inhibition by native VIP of specific ^{125}I -VIP binding to particulate preparation

Conditions are as described in the Experimental section. Value are means $\pm$ S.E.M. for seven separate experiments.

Fig. 4. Peptide specificity of covalent labelling of HT-29 cell homogenate proteins by ^{125}I -VIP after SDS/polyacrylamide-gel electrophoresis

Conditions are as described in the Experimental section. Particulate preparations were incubated with ^{125}I -VIP and other peptides, then treated with 1 mM-DTSP. Lane (a) shows the autoradiographic pattern of homogenate incubated with ^{125}I -VIP alone; lane (b), with 1 μM native VIP; lane (c), with 1 μM -glucagon; lane (d), with 1 μM -octapeptide of cholecystokinin. Calibrating proteins were: myosin (M_r 200000), phosphorylase *b* (M_r 92000), bovine serum albumin (M_r 68000), ovalbumin (M_r 43000), chymotrypsinogen (M_r 25700), lactoglobulin (M_r 18400) and cytochrome *c* (M_r 12300).

We have performed cross-linking experiments in order to identify the VIP receptor in HT-29 cells at the molecular level. When HT-29 cell homogenates are incubated with ^{125}I -VIP at equilibrium, washed free of unbound VIP and treated with 1 mM cross-linker (DTSP), polyacrylamide-gel electrophoresis of proteins reveals, after autoradiography, two major bands corresponding

Fig. 5. Dose-response of inhibition by native VIP (○) and VIP-(2-28)-peptide (●) of the covalent labelling of the M_r -66000 ^{125}I -VIP-protein complex

Conditions are as described in the Experimental section. Particulate preparations were incubated with ^{125}I -VIP and various concentrations of native VIP or VIP-(2-28)-peptide, then treated with $1\ \mu\text{M}$ -DTSP. After electrophoresis, the resulting autoradiographs were scanned as described by Laburthe *et al.* (1984). The peak areas corresponding to the ^{125}I -VIP-protein complex of M_r 66000 were integrated. Results are expressed as the percentage of the initial peak area for particulate preparation incubated with ^{125}I -VIP alone. Each point is the mean $\pm$ S.E.M. of three experiments.

to ^{125}I -VIP-protein complexes of M_r 66000 and 16000 (Fig. 4, lane *a*). The specificity of labelling of these bands is tested by adding native VIP ($0.1\ \mu\text{M}$) with ^{125}I -VIP. The labelling of the M_r -66000 component is completely abolished by native VIP, whereas that of the M_r -16000 component is poorly affected (Fig. 4, lane *b*). This indicates that the M_r -66000 component is involved in the specific binding of VIP to HT-29 cells, whereas the M_r -16000 component is not specifically labelled by ^{125}I -VIP. As shown in Fig. 4 (lanes *c* and *d*), the addition of $1\ \mu\text{M}$ glucagon or $1\ \mu\text{M}$ -octapeptide of cholecystokinin does not affect the labelling of the M_r -66000 component, indicating its peptide specificity. The concentration-dependence of VIP binding to the M_r -66000 component is tested by adding increasing concentrations of native VIP with ^{125}I -VIP. Integration of the peak obtained by micro-densitometric scanning of autoradiographs (Laburthe *et al.*, 1984) shows that the covalent labelling of the M_r -66000 component is inhibited by native VIP in the concentration range 0.1 – $10\ \text{nM}$ (Fig. 5), half-maximal inhibition occurring at $0.6 \pm 0.1\ \text{nM}$ (mean $\pm$ S.E.M. for four experiments). The structural requirement of the M_r -66000 component is tested with the most potent VIP agonist, i.e. VIP-(2-28)-peptide (see Fig. 2). VIP-(2-28)-peptide is about 100 times less potent than VIP in inhibiting the covalent labelling of the M_r -66000 component (Fig. 5). This compares well with the relative potency of the two peptides in competing with ^{125}I -VIP for binding to HT-29 cells.

DISCUSSION

The present paper indicates that the human colon adenocarcinoma cell line HT-29 in culture expresses a single population of high-affinity binding sites for the

neuropeptide VIP. The dissociation constant of these sites, i.e. $0.12\ \text{nM}$, compares well with the ED_{50} of VIP in stimulating cyclic AMP production, i.e. $0.3\ \text{nM}$ (Laburthe *et al.*, 1978*b*) or protein kinase activity, i.e. $0.1\ \text{nM}$ (Marvaldi *et al.*, 1979) or glycogenolysis, i.e. approx. $0.3\ \text{nM}$ (Rousset *et al.*, 1981) in HT-29 cells. If we assume that one molecule of VIP (M_r 3326) is bound per molecule of receptor, the cross-linking experiments indicate that VIP is covalently bound to a HT-29 cells protein with an average M_r of 63000. This protein has the properties expected for the high-affinity VIP receptor that mediates the activation of adenylate cyclase. The cross-linking of ^{125}I -VIP to this protein is inhibited by low concentrations (0.1 – $10\ \text{nM}$) of native VIP (see Fig. 5), within the dose-response curve for both the stimulation of cyclic AMP production (Laburthe *et al.*, 1978*b*) and the saturation of VIP-binding site (see Figs. 2 and 3). Furthermore, the M_r -63000 protein is specific, since it is not altered by peptides such as glucagon and octapeptide of cholecystokinin, and is able to discriminate the VIP agonist VIP-(2-28)-peptide. This protein has thus the properties of apparent affinity, specificity and discriminating power toward agonists that are characteristic of the VIP receptor in HT-29 cells.

In good agreement with Scatchard analysis of binding data in HT-29 cells, which indicates only one population of VIP-binding sites, the covalent labelling of HT-29 cells by ^{125}I -VIP reveals only one specific M_r -63000 VIP-binding protein. This contrasts markedly with the observations made in normal human colonic cells, where Scatchard analysis revealed two classes of binding sites, a high-affinity/low-capacity site and a low-affinity/high-capacity site (Broyart *et al.*, 1981), corresponding to two different VIP-binding proteins identified by covalent cross-linking as a M_r -63000 protein and a M_r -30000 protein respectively (Couvineau & Laburthe, 1985). The reason why the low-affinity binding site is not observed in HT-29 cells is unknown. What is clear, however, is that the molecular size of the VIP receptor in HT-29 cells, i.e. 63000 Da, is identical with that of the high-affinity VIP-binding site mediating adenylate cyclase activity by VIP in normal human colonic cells (Couvineau & Laburthe, 1985). This molecular size is in contrast different from that determined in rat intestinal epithelial cells, i.e. 73000 Da (Laburthe *et al.*, 1984), and appears be typical of the human intestine, either normal or transformed. As far as the molecular size of the VIP receptor is concerned, it thus appears that HT-29 cells retain their species specificity in the expression of VIP receptors. They also retain their species specificity in another feature, the structural requirement of the VIP-recognition site. Indeed, the relative affinities of the different VIP agonists, i.e. $\text{VIP} > \text{VIP-(2-28)-peptide} > \text{hGRF} > \text{PHI} > \text{secretin}$, in HT-29 cells is identical with that described previously in plasma membranes from normal human colonic cells, but contrasts markedly with that described in rat intestinal epithelium, i.e. $\text{VIP} > \text{PHI} > \text{VIP-(2-28)-peptide} > \text{secretin} > \text{hGRF}$ (Laburthe *et al.*, 1983; Couvineau *et al.*, 1984). Like VIP receptors in rat or human intestine (Couvineau *et al.*, 1984), VIP receptors in HT-29 cells are very susceptible to the absence of the *N*-terminal histidine [as in VIP-(2-28)-peptide], since the deletion of this amino acid results in a 100-fold decrease in affinity (see Fig. 2). Furthermore, the very low affinity of PHI and secretin for VIP receptors in HT-29 cells contrasts with their much higher affinity

for rat VIP receptors and further confirms that the VIP receptor in the human has evolved towards a high degree of specificity of its recognition site for the members of the so-called VIP-secretin family (Laburthe *et al.*, 1983, 1985).

In conclusion, the present work indicates a M_r -63 000 protein is involved in the specific binding of VIP to one single population of high-affinity sites in the human colon adenocarcinoma cell line HT-29. These receptors display structural requirements and molecular size identical with those of the high-affinity VIP receptor observed in normal human colonic cells. This further indicates the validity of the HT-29 cell line for studying VIP-receptor structure and function.

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