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Characterization of VIP- and Helodermin-Preferring Receptors on Human Small Cell Lung Carcinoma Cell Lines

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LUIS, J. AND S. I. SAID. *Characterization of VIP- and helodermin-preferring receptors on human small cell lung carcinoma cell lines*. PEPTIDES **11**(6) 1239–1244, 1990.—We investigated the molecular and pharmacologic characteristics of VIP receptors on two human SCLC cell lines: NCI-N592 and NCI-H345. With NCI-N592 cell, the order of potency of VIP-related peptides in inhibiting ^{125}I-VIP binding and in stimulating cAMP production was typical of the human VIP receptor. By covalent cross-linking, a polypeptide of Mr 62,300 was obtained. Conversely, the behavior of NCI-H345 cell line was totally different: helodermin was the most potent peptide, VIP and PHI were equipotent, while hGRF and secretin were totally ineffective. These results suggest that NCI-N592 cells possess a typical VIP receptor while NCI-H345 cells possess a helodermin-preferring receptor, and that the natural target of helodermin might not be the VIP receptor.

VIP VIP receptor Helodermin-like peptides Helodermin receptor Small cell lung carcinoma SCLC cell lines Molecular characterization Cross-linking

THE vasoactive intestinal peptide (VIP), initially isolated from porcine intestine (22,31), is a 28 amino acid residue neuropeptide that is widely distributed in the body. Specific receptors for VIP have been found in a large variety of tissues and cell lines. In most cases studied so far, the binding of VIP to its receptor leads to an increase in cAMP level (3, 6, 20). VIP might also act through the inositol phosphate system, since it triggers the degradation of phosphoinositol in selected tissues (2). The first results on the structure of the VIP receptor were obtained using the covalent cross-linking method in rat enterocytes (15). This technique led afterwards to the molecular characterization of the VIP binding sites from numerous tissues and cell lines [for a review, see (17)]. The VIP receptor has been characterized as a glycoprotein containing a large amount of carbohydrates (8, 9, 23).

Helodermin, a 36 amino acid residue peptide related to VIP and secretin, was purified from the venom of the Gila monster lizard (*Heloderma suspectum*) (12). Helodermin-like immunoreactivity has also been shown in mammalian tissues (11,28). Moreover, Robberecht *et al.* (29,30) have recently described a new type of "VIP receptor," with higher affinity for helodermin than for VIP and no affinity for secretin or hGRF. This subtype of VIP receptor has been called the helodermin/VIP receptor or the helodermin-preferring receptor.

Small cell lung carcinomas (SCLC), as well as cell lines established from these tumors, secrete numerous peptide hormones including adrenocorticotropin (ACTH), bombesin, calcitonin, somatostatin, prolactin and glucagon (1,33). High levels of bombesin/gastrin releasing peptide-like immunoreactivity are found in most of SCLC cell lines, suggesting that these peptides may be an autocrine factor for SCLC growth (5). Recently, binding sites for VIP have been localized on plasma membranes from human SCLC cell lines (32). Further, preliminary studies have suggested that hormone secretion by SCLC cells may be controlled by the level of the intracellular second messenger cAMP (34). The importance of cAMP in mediating the action of VIP has been well documented and VIP has been shown to elevate cAMP levels in SCLC cell lines (13). This increase in intracellular cAMP level leads to a stimulated release of bombesin-like peptides from SCLC cell lines, as well as to an increased plasma bombesin-like peptides level in patients with extrapulmonary small cell lung cancer (13). Investigation of the actions of VIP and characterization of its receptor on these cell lines could therefore greatly advance our knowledge of the biology of small cell lung carcinomas.

We report here on the characterization of the VIP receptor on human SCLC cell line NCI-N592, and on the presence of a receptor with higher affinity for helodermin than for VIP or related peptides on a second SCLC cell line, NCI-H345.

METHOD

Materials

Purified porcine VIP was provided by Professor V. Mutt

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ABBREVIATIONS

BSA	bovine serum albumin
cAMP	cyclic 3′-5′ adenosine monophosphate
DME medium	Dulbecco's modified Eagle's medium
DSP	disuccinimidyl propionate
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hGRF	human growth hormone releasing factor(1–44)
IBMX	3-isobutyl-1-methyl xantine
PHI	peptide histidine isoleucine amide
PHV	peptide histidine valine amide
PMSF	phenylmethylsulphonyl fluoride
SCLC	small cell lung carcinoma
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
VIP	vasoactive intestinal peptide
^{125}I-VIP	monoiodinated ^{125}I-vasoactive intestinal peptide

(Karolinska Institutet, Stockholm, Sweden). Helodermin, helospectins I and II, porcine PHI, human PHV, secretin and glucagon were purchased from Peninsula Laboratories Inc. (San Carlos, CA). Human GRF(1–44) (hGRF), Iodogen and fetal bovine serum were from Sigma (St. Louis, MO), electrophoretic chemicals and Mr standards from BioRad Laboratories (Richmond, CA), and RPMI 1640 medium from Gibco (Grand Island, NY).

VIP was iodinated with ^{125}INa from NEN (Boston, MA) by the lactoperoxidase (19) or the Iodogen method (10). Monoiodinated VIP (^{125}I-VIP) was purified as previously described (19,24).

Cell Culture

The human SCLC cell lines NCI-H345 and NCI-N592 (4) were kindly provided by Dr. Adi F. Gazdar (NCI, Bethesda, MD) and were routinely cultured in RPMI 1640 medium containing 5% heat-inactivated fetal bovine serum in a humidified atmosphere of air/CO_2 (19:1).

cAMP Extraction and Measurement

cAMP extraction was performed as already described (20). Briefly, SCLC cells were incubated ($0.8–1.4 \times 10^6$ cells/ml) in 0.5 ml DME medium pH 7.3, containing 2% bovine serum albumin (BSA), 0.6 mM IBMX and VIP or other peptides. After incubation for 10 min at 25°C, 2.5 ml methanol was added to stop the reaction and to extract cAMP. Samples were then centrifuged for 5 min at $3000 \times g$. The resulting pellet was washed with 1 ml methanol and the washing solution added to the supernatant of each sample. Combined solutions were evaporated to dryness and residues dissolved in 1 ml cAMP assay buffer (NEN). After centrifugation, supernatants were assayed for cAMP using ^{125}I-cAMP (NEN).

Membrane Preparation

Membranes from SCLC cells were prepared as described (32) with slight modifications. Cells were resuspended at $20–50 \times 10^6$ cells/ml in 20 mM Tris buffer pH 7.8, containing 1 mM EGTA, 1 mM $MgCl_2$ and 150 μM PMSF. The cells were then homogenized using a Dounce homogenizer. After 20 strokes the mixture was centrifuged at $1500 \times g$ for 10 min at 4°C. The supernatant was saved and the pellet resuspended, homogenized and centrifuged. Both supernatants were combined and centrifuged for 15 min at $20,000 \times g$. The pellet was then resuspended in 10 mM Hepes pH 7.4, containing 104 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$ and 1 mM KH_2PO_4 (membrane buffer).

^{125}I-VIP Binding to Intact Cells

SCLC cells were washed twice with ice-cold phosphate-buffered saline containing 0.1% BSA (PBS/BSA). Cells were incubated with 0.05 nM ^{125}I-VIP in 0.2 ml DME medium containing 15 mM Hepes pH 7.4, 150 μM PMSF, 1% BSA and 0.1% bacitracin for 3 hr at 13°C. The cell suspension was then layered over 1 ml ice-cold PBS/BSA and centrifuged for 20 sec in an Eppendorf microfuge. After washing with 1 ml PBS/BSA, the radioactivity bound to the cells was counted in a γ-radiation spectrometer (Beckman). Specific binding was calculated as the difference between the mean of determinations of total binding and binding in the presence of 1 μM native VIP.

^{125}I-VIP Binding to Plasma Membranes

Forty to 60 μg of membrane protein were incubated with 0.05 nM ^{125}I-VIP in 0.2 ml of membrane buffer containing 150 μM PMSF, 1% BSA and 0.1% bacitracin for 25 min at 25°C. The suspension was then processed as for the cells except that the centrifugation was for 1 min.

Covalent Cross-Linking

Covalent cross-linking was performed as previously described (21). Briefly, after ^{125}I-VIP binding, cells were rinsed twice with phosphate-buffered saline and resuspended in 1 ml 60 mM Hepes buffer pH 8.0, containing 150 mM NaCl (Hepes buffer). The reaction was initiated by addition of 20 μl 100 mM disuccinimidyl propionate (DSP) and allowed to proceed for 20 min at room temperature. The reaction was stopped by adding 0.5 ml Hepes buffer containing 60 mM ammonium acetate. Labeled cells were then solubilized for 20 min on ice with 160 μl 100 mM phosphate buffer pH 6.1, containing 5 mM EDTA, 5 mM $MgCl_2$ 1% Triton X100 and 0.1% SDS. Proteins were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (16).

SDS/Polyacrylamide Gel Electrophoresis

Ten percent SDS-PAGE was run under nonreducing conditions in slab gel, 3.5 mm thick. After electrophoresis the gels were stained with Coomassie blue R-250, destained and dried. Gels were then exposed to Kodak AR-X-Omat film for 2–3 weeks. Calibration proteins were: myosin (Mr 200,000), β-galactosidase (Mr 116,250), phosphorylase b (Mr 97,400), bovine serum albumin (Mr 66,200), ovalbumin (Mr 42,700), carbonic anhydrase (Mr 31,000), soybean trypsin inhibitor (Mr 21,500) and lysozyme (Mr 14,400).

RESULTS

Specific Binding of ^{125}I-VIP to SCLC Cells and Membranes

In competition experiments between ^{125}I-VIP and native VIP (Fig. 1), native VIP inhibited ^{125}I-VIP binding to NCI-N592 cells in the 0.03 nM–1 μM range. Half-maximal inhibition of ^{125}I-VIP binding (IC_{50}) was observed for 2.2 ± 0.5 nM native VIP (mean ± SEM from three separate experiments). Scatchard analysis of the data (Fig. 1, inset) was curvilinear, suggesting the presence of two classes of VIP binding sites on NCI-N592 cells: a high affinity binding site (dissociation constant, $K_d = 0.77 \pm 0.19$ nM) and a low affinity binding site ($K_d = 17.6 \pm 4.4$ nM). The

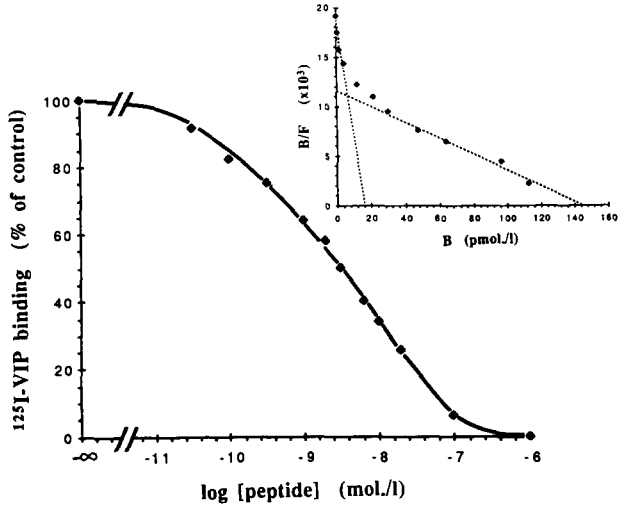

FIG. 1. Competitive displacement of ^{125}I-VIP from NCI-N592 cells by native VIP. NCI-N592 cells were incubated with monoiodinated VIP in the presence of increasing concentrations of native VIP, as described in the experimental section. Results are from one experiment representative of three performed in triplicate. Scatchard analysis of the data is shown in the inset. B, bound ligand, F, free ligand.

number of binding sites was 1060 ± 80 and $12{,}900 \pm 3100$ per cell, respectively.

The specificity of VIP binding to its receptor was investigated by competition experiments between ^{125}I-VIP and VIP-related peptides on plasma membranes from NCI-N592 cells. The order of potency of the different peptides in inhibiting monoiodinated ^{125}I-VIP binding was: VIP ($IC_{50} = 1.0$ nM) >> helodermin ($IC_{50} = 20$ nM) > PHI ($IC_{50} = 220$ nM) > hGRF ≥ secretin ($IC_{50} \approx 1000$ nM) (Fig. 2). Glucagon did not inhibit ^{125}I-VIP binding in concentrations of up to 1 μM.

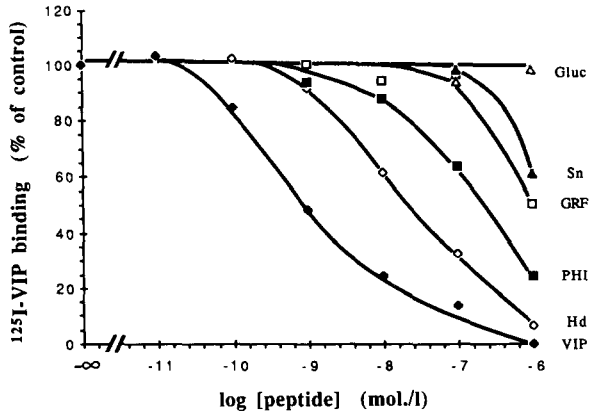

FIG. 2. Competition for ^{125}I-VIP binding to NCI-N592 plasma membranes by VIP-related peptides. NCI-N592 cells were incubated with ^{125}I-VIP in the presence of increasing concentrations of VIP (◆), helodermin (◇), PHI (■), GRF (□), secretin (▲) or glucagon (△). Results are expressed as the percentage of specifically bound radioactivity. Data are means of four independent experiments performed in triplicate. Hd: helodermin, Sn: secretin, Gluc: glucagon.

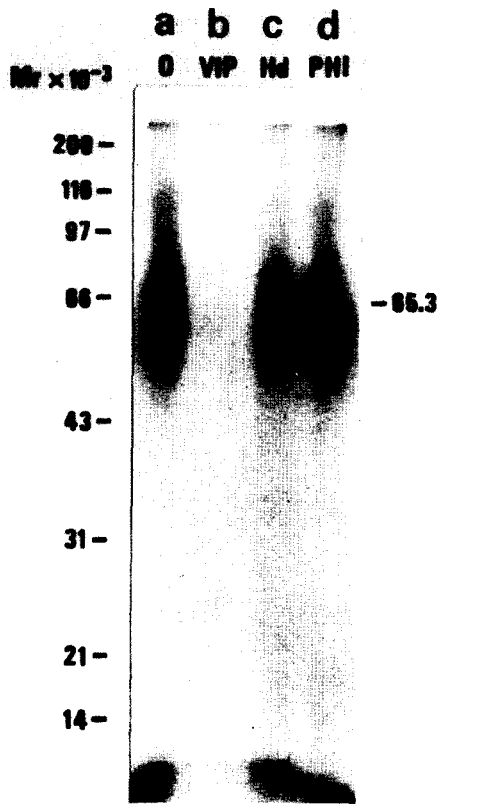

FIG. 3. Cross-linking of the VIP binding polypeptides of NCI-N592 cells by DSP. NCI-N592 cells were incubated with ^{125}I-VIP for 3 hr at 13°C in the absence (lane a) or the presence of 1 μM of native VIP (lane b), 1 μM helodermin (lane c) or 1 μM PHI (lane d), and were treated with 2 mM DSP. The labeled peptides were then solubilized and analyzed by SDS-PAGE.

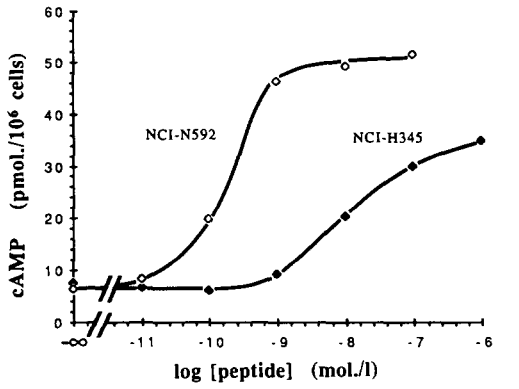

FIG. 4. Dose-dependent effect of VIP on cAMP accumulation in NCI-N592 and NCI-H345 cells. NCI-N592 cells (◇) and NCI-H345 cells (◆) were incubated in DME medium containing 2% BSA and 0.6 mM IBMX for 10 min at 25°C in the presence of increasing concentrations of VIP. The reaction was stopped and cAMP extracted and measured as described in the experimental section. Data are means of four independent experiments performed in triplicate.

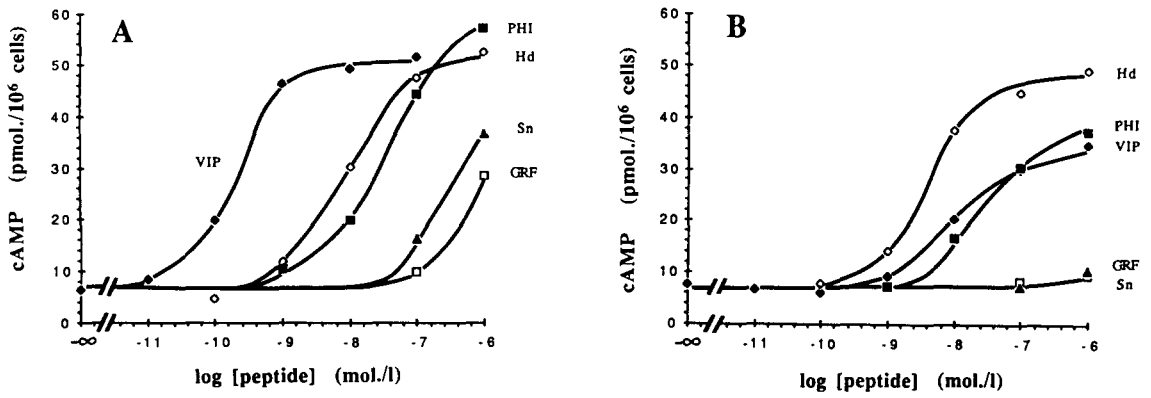

FIG. 5. Dose-dependent effect of VIP and VIP-related peptides on cAMP accumulation in NCI-N592 and NCI-H345 cells. NCI-N592 cells (A) and NCI-H345 cells (B) were incubated in DME medium containing 2% BSA and 0.6 mM IBMX for 10 min at 25°C in the presence of increasing concentrations of VIP (◆), helodermin (◇), PHI (■), secretin (▲) or GRF (□). The reaction was stopped and cAMP was extracted and measured as described in the experimental section. Data are means of three independent experiments performed in triplicate. Hd: helodermin, Sn: secretin.

Molecular Characterization of the VIP Binding Sites of NCI-N592 Cells

The molecular identification of the VIP binding sites on intact NCI-N592 cells was carried out by cross-linking experiments. NCI-N592 cells were incubated with ^{125}I-VIP, washed to remove the unbound ligand and treated with the cross-linking reagent DSP. After solubilization, the labeled material was analyzed by SDS-PAGE under nonreducing conditions. The resulting autoradiograph of the gel showed a labeled complex with a Mr of 65,300 ± 1900 (n = 5). A minor complex with a Mr of 110–120,000 was also inconsistently found.

The specificity of the labeling was investigated with VIP and VIP-related peptides. Labeling of the peptide was completely abolished when the incubation step was performed in the presence of 1 μM native VIP (Fig. 3, lane b). Incubation in the presence of 1 μM helodermin or PHI significantly reduced the extent of the labeling (Fig. 3, lanes c and d, respectively). Conversely, incubation with 1 μM glucagon did not affect the labeling of the peptide (data not shown).

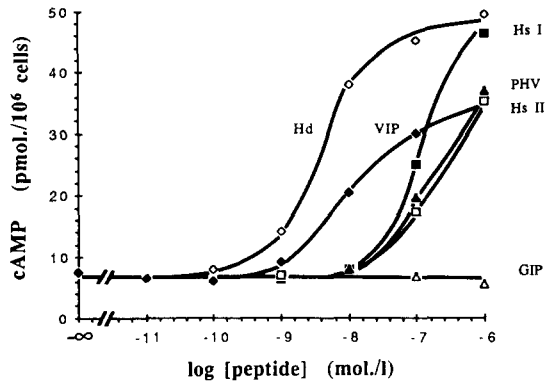

FIG. 6. Dose-response effect for cAMP accumulation induced by VIP-related peptides in NCI-H345 cells. NCI-H345 cells were incubated in DME medium containing 2% BSA and 0.6 mM IBMX for 10 min at 25°C in the presence of increasing concentrations of VIP (◆), helodermin (◇), PHV (▲), helospectin I (■), helospectin II (□) or GIP (△). The reaction was stopped and cAMP extracted and measured as described in the experimental section. Data are means of three independent experiments performed in triplicate. Hd: helodermin, Hs I: helospectin I, Hs II: helospectin II.

cAMP Accumulation in SCLC Cell Lines in Response to VIP

The cAMP production by NCI-N592 and NCI-H345 cells in response to VIP was investigated. In the presence of IBMX, VIP increased cAMP levels in both cells, but with markedly different potencies and efficacies (Fig. 4). Maximal responses were 8.2 and 4.8 times the basal level for NCI-N592 and NCI-H345 cells, respectively. Moreover, with NCI-N592 cells, maximal and half-maximal effects were observed for VIP concentrations of 10 nM and 0.2 nM, respectively, whereas with NCI-H345 cells, the corresponding values were 100 times higher, at 1 μM and 20 nM.

cAMP Accumulation in SCLC Cell Lines in Response to VIP-Related Peptides

We investigated the effects of different VIP-related peptides on cAMP production in both SCLC cell lines. All peptides were effective in stimulating cAMP accumulation in the NCI-N592 cell line (Fig. 5A). The order of potency was the same as for the inhibition of ^{125}I-VIP binding: VIP >> helodermin > PHI > secretin > hGRF. However, with the NCI-H345 cell line, this order was totally different (Fig. 5B): helodermin was the most potent peptide, VIP and PHI were equipotent, while hGRF and secretin were totally ineffective.

We also tested the other members of this family of peptides on NCI-H345 cells (Fig. 6). Helodermin was still the most potent peptide in stimulating cAMP production by this cell line.

DISCUSSION

Most of the information available on the VIP receptor in the lung has been obtained with membranes from whole tissue (7, 25–27). Recent studies have reported specific binding of ^{125}I-VIP on plasma membranes from various human lung cancer cell lines (32). In the present study we demonstrate the existence of two classes of VIP binding sites in intact NCI-N592 cells by competition experiments. Scatchard analysis of the data showed that each NCI-N592 cell possesses 1060 high affinity binding sites (K_d = 0.77 nM) and 12,900 low affinity binding sites (K_d = 17.6 nM). The competition experiments performed between ^{125}I-VIP and VIP-related peptides on plasma membranes from these cells

showed the following order of potency: VIP >> helodermin > PHI > hGRF ≥ secretin. Glucagon had no effect on ^{125}I-VIP binding. This order of efficacy is the same as that observed with other human cells (14,18) and seems to be typical of the human VIP receptor.

When covalent cross-linking experiments using the homobifunctional reagent disuccinimidyl propionate (DSP) were performed on NCI-N592 cells, a complex of Mr 65,300 was obtained. The labeling was specific and significantly reduced by incubation in the presence of helodermin or PHI, but not in the presence of glucagon. Assuming one molecule of VIP linked to the complex, the molecular mass of the VIP binding polypeptide can be estimated at 62,300. This molecular mass is similar to the values obtained with human lung and with most of the human tissues and cell lines (Mr 60,000–65,000) [for a review see (17)], but differs from that of rat lung membranes (Mr 53,000) (7).

As in most cases described so far, VIP stimulated cAMP synthesis in SCLC cells, but in this case the potency and efficacy varied greatly depending on the cell line. With NCI-H345 cells the maximal and half-maximal responses were observed for VIP concentrations 100 times higher than with NCI-N592 cells. To check if in NCI-H345 cells the effect of VIP on cAMP accumulation is mediated by the VIP receptor, we investigated the effect of VIP-related peptides on cAMP production by both cell lines. As expected, with NCI-N592 cell the order of potency was the same as for the inhibition of ^{125}I-VIP binding, i.e., VIP >> helodermin > PHI > secretin > hGRF. On the other hand, the behavior of NCI-H345 cell line was totally different: helodermin was the most potent peptide in stimulating cAMP production, VIP and PHI were equipotent, while hGRF and secretin were totally ineffective.

Using ^{125}I-helodermin, Robberecht *et al.* (29,30) recently described a novel subtype of the "VIP receptor" on human SUP-T1 lymphoblasts. This receptor is characterized by a higher affinity for helodermin than for VIP, by equal potency of VIP and PHI and by the absence of recognition of secretin and human GRF. These pharmacologic differences from the "typical" human VIP receptor led the authors to propose the term "helodermin/VIP receptor" or "helodermin-preferring receptor." Thus, based on pharmacologic data, it seems that NCI-N592 cells possess a typical VIP receptor while NCI-H345 cells possess a helodermin-preferring receptor.

Among all known VIP-related peptides, helodermin was the most potent in stimulating cAMP production by NCI-H345 cell line. Since its discovery, this peptide, originally purified from lizard venom, was thought to act through VIP or secretin receptors. However, secretin had no effect on cAMP production by this cell line, and VIP had little effect, suggesting that the natural target for helodermin might not be the VIP receptor nor the secretin receptor. The existence of a helodermin-preferring receptor suggests that helodermin might act through a receptor for another VIP-related peptide. The presence of helodermin-like immunoreactivity in mammalian tissues (11,28) is consistent with this concept.

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