Photoaffinity labelling of the vasoactive-intestinal-peptide-binding site on intact human colonic adenocarcinoma cell line HT29-D4

Synthesis and use of photosensitive vasoactive-intestinal-peptide derivatives

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N-Hydroxysuccinimidyl 4-azidobenzoate, a u.v.-sensitive heterobifunctional reagent, was used to synthesize photoreactive derivatives of the vasoactive intestinal peptide (VIP). Products of the reaction were purified by reverse-phase h.p.l.c. Three 4-azidobenzoyl-VIP (4-AB-VIP) derivatives were able to compete with monoiodinated ^{125}I -VIP with an apparent K_D of 2.5, 6.3 and 12.5 nM compared with 0.6 nM for native VIP. H.p.l.c.-purified mono[^{125}I]iodinated VIP was used to synthesize 4-AB- ^{125}I -VIP derivatives. They were used to photoaffinity-label the VIP-binding site of HT29-D4 cells, a clone derived from the human colonic adenocarcinoma cell line HT29. Only one polypeptide, of M_r 70000 $\pm$ 5000 (mean $\pm$ s.d.) was specifically labelled. The M_r of the component thus characterized was slightly higher than that of the major species (M_r 67000) labelled after cross-linking experiments using ^{125}I -VIP, conventional homobifunctional reagents and HT29 cells. Nevertheless, the specificity and extent of glycosylation of these two components were identical. These new photosensitive VIP derivatives should be useful tools with which to investigate further VIP-receptor structure and metabolism.

INTRODUCTION

The vasoactive intestinal peptide (VIP), a highly basic single-chain polypeptide of 28 residues (M_r 3326), is a neuropeptide which belongs to the glucagon–secretin family. First isolated from porcine intestine (Said & Mutt, 1972), VIP has been found to be widely distributed in the whole organism, where it regulates many biological activities at the peripheral- and the central-nervous-system level (for a review, see Said, 1984). Specific receptors for VIP, functionally linked to adenylate cyclase, have been described in various mammalian tissues, including brain (Marley & Emson, 1982), liver (Desbuquois, 1974), intestine (Laburthe *et al.*, 1979) and pancreas (Christophe *et al.*, 1976).

Receptors of high affinity for VIP have been also characterized in the human colonic adenocarcinoma cell line HT29 (Fogh & Trempe, 1975), where VIP is a potent activator of the cyclic AMP production system (Laburthe *et al.*, 1978). The increased level of cyclic AMP triggers a subsequent activation of both cyclic AMP-dependent protein kinases and cyclic AMP phosphodiesterases (Marvaldi *et al.*, 1979; Mangeat *et al.*, 1981).

Molecular identification of VIP-binding sites of HT29 cells has been carried out by covalent cross-linking experiments, using the homobifunctional reagent dithio-bis(succinimidyl propionate), on cell homogenates (Couvineau *et al.*, 1985), crude membranes (Marvaldi *et al.*, 1986) or intact cells (Muller *et al.*, 1985a). In

these studies, assuming only one ^{125}I -VIP molecule cross-linked per polypeptide, a species of M_r 64000 (67000–3000) was characterized. This component behaved like a high-affinity binding site for VIP. On intact cells additional minor species of M_r 31000 and M_r 117000 were also labelled.

Recently, covalent ^{125}I -VIP–receptor complexes were used to demonstrate the glycoprotein nature of VIP-binding sites on HT29-D4 cells (El Battari *et al.*, 1987), a clone of the HT29 cell line (Fantini *et al.*, 1986). The M_r -64000 species is a glycoprotein with sialic-acid-containing N-linked oligosaccharide side chains. The glycoprotein moiety accounts for 20 kDa.

Cross-linking experiments using homobifunctional reagents have two major disadvantages: (1) incubation of intact cells or membranes in the presence of high concentrations of cross-linking reagents may lead to covalent links between the radioiodinated ligand and proteins different from the true binding site or between receptor and neighbouring proteins (Ji, 1983); (2) incubation of intact cells in the presence of cross-linking reagents could induce perturbations of the dynamics of the membrane and therefore obviate studies on receptor cycling in the cell.

Photoaffinity labelling is a much more attractive approach with which to study the molecular structure (Mazella *et al.*, 1985; Shin & Ji, 1985) and the dynamics (Berhanu *et al.*, 1982; Oka *et al.*, 1985; Huecksteadt *et al.*, 1986) of receptors, because covalent attachment of

Abbreviations used: VIP, vasoactive intestinal peptide; PAGE, polyacrylamide-gel electrophoresis; DMF, *N,N*-dimethylformamide; HSAB, *N*-hydroxysuccinimidyl 4-azidobenzoate; RP-h.p.l.c. reverse-phase h.p.l.c.; PBS, Dulbecco phosphate-buffered saline [(mg/l) NaCl, 8000; KCl, 200; $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 1440; KH_2PO_4 , 200, pH 7.3]; Endo F, endo- β -acetylglucosaminidase F; 4-AB-VIP, 4-azidobenzoyl-vasoactive intestinal peptide; BSA, bovine serum albumin.

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the ligand to its receptor essentially occurs at the binding-site level. Thus non-specific labelling is prevented and the dynamic capacity of membrane is preserved.

In the present study, 4-azidobenzoyl (4-AB) derivatives of monoiodinated VIP were synthesized and used successfully to photoaffinity-label the VIP receptor on intact HT29-D4 cells.

EXPERIMENTAL

Materials

Synthetic porcine VIP, VIP-(10–28)-peptide fragment, glucagon and bovine serum albumin were purchased from Sigma (St. Louis, MO, U.S.A.). *NN*-Dimethylformamide (DMF) and *N*-hydroxysuccinimidyl 4-azidobenzoate (HSAB) were obtained from Pierce Chemical Co. (Rockford, IL, U.S.A.). Dulbecco's modified Eagle's medium was from Gibco (Cergy Pontoise, France). Foetal-calf serum was from Eurobio (Paris, France). Carrier-free Na^{125}I was from Amersham International (Amersham, Bucks., U.K.). Electrophoresis chemicals and M_r standards were from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Endo- β -acetylglucosaminidase F (Endo F) was generously given by Dr. J. Elder and Dr. S. Alexander (Research Institute of Scripps Clinic, La Jolla, CA, U.S.A.). Other chemicals were of the highest purity commercially available, and when necessary, of h.p.l.c. grade.

Cell culture

HT29-D4 was a clone of the human colonic adenocarcinoma cell line HT29 (Fogh & Trempe, 1975) (a gift of Dr. Zweibaum, Paris, France) obtained in our laboratory (Fantini *et al.*, 1986). HT29-D4 and Madin Darby canine kidney epithelial cells (MDCK cells) were routinely cultured in Dulbecco's modified Eagle's medium containing glucose (4.5 g/l) and 10% (v/v) foetal-calf serum in a humidified atmosphere of air/ CO_2 (19:1). Subcultures were obtained by harvesting exponentially growing cells with 0.53 mM-EDTA containing 0.05% trypsin in PBS. Medium was changed daily.

Iodination procedure

A 5 μg portion of synthetic porcine VIP was iodinated by the chloramine- τ method (Hunter & Greenwood, 1962), using 0.5 M-sodium phosphate buffer, pH 7.0, in a final volume of 70 μl . Radioiodination was performed by adding 2 mCi of carrier-free Na^{125}I and 5 μl of a 1 mg/ml solution of chloramine- τ . The reaction was allowed to proceed for 30 s at room temperature and was terminated by the addition of 20 μl of a 0.6 mg/ml solution of sodium metabisulphite.

Monoiodinated VIP purification procedure

We used the method described by Martin *et al.* (1986) with some minor modifications. The iodination mixture was diluted with 1 ml of 10% (v/v) acetonitrile in 150 mM (12 mS)-ammonium formate buffer, pH 2.7, and applied to a SEP-PAK cartridge (Waters) pre-equilibrated with 2 ml of the same buffer. Unbound material was washed away with 20 ml of 10% acetonitrile in formate buffer. Retained peptides were eluted with 1 ml of 60% acetonitrile in the formate buffer. The volume of the eluted product was decreased to approx. 300 μl under

a stream of N_2 . Further purifications were performed by RP-h.p.l.c. The concentrated SEP-PAK eluate was injected into an Ultrosphere Octyl column (Beckman; ref. 235332) pre-equilibrated with 25% acetonitrile in formate buffer. After 3 min a linear gradient of acetonitrile (25–40% over 30 min) was applied with a flow rate of 1.5 ml·min⁻¹. Fractions (750 μl each) were collected. The radioactivity in each fraction was determined by counting 5 μl portions in a γ -radiation spectrometer (Packard). Fractions corresponding to monoiodinated VIP were pooled, and the acetonitrile was evaporated off under a stream of N_2 . Bovine serum albumin (BSA) was added (final concn. 12 mg/ml) to prevent adsorption and degradation of VIP. Monoiodinated VIP was then divided into 2 μCi portions, freeze-dried and kept at -48°C until used.

Synthesis and analysis of VIP derivatives

4-AB-VIP. A 5 μl portion of HSAB (3 mM in anhydrous DMF) was added to 50 μg of native VIP dissolved in 50 μl of 50 mM-Tris/HCl, pH 8.6. The reaction was allowed to proceed in the dark for 30 min at room temperature.

Mono[^{125}I]iodinated 4-AB-VIP. In routine experiments, a portion (2 μCi) of BSA-containing monoiodinated VIP was dissolved in 110 μl of 50 mM-Tris/HCl, pH 8.6 (final concn. of BSA 5 mg/ml). HSAB, dissolved in 11 μl of anhydrous DMF, was then added (Molar ratio of reagent to total reactive amino groups of monoiodinated VIP plus BSA 5:1). Molar ratios ranging from 2:1 to 10:1 have been used without any modification in the M_r of the subsequently labelled protein. After 30 min the reaction was quenched by a 20-fold dilution with Dulbecco's modified Eagle's medium containing 15 mM-Hepes (pH 7.4)/150 mM-phenylmethanesulphonyl fluoride/1% BSA/0.1%-bacitracin (binding medium).

Analytical procedure. Analysis of reaction mixtures was performed by RP-h.p.l.c. as described for purification of monoiodinated VIP, except that a linear gradient of acetonitrile (10–50% over 40 min) was applied.

Amino acid analysis. A 1 nmol portion of each freeze-dried peptide sample was hydrolysed in 400 μl of 6 M-HCl at 150°C for 2 h and analysed by standard methods on a Beckman system 6300 amino acid analyser.

Binding and photoactivation of mono[^{125}I]iodinated 4-AB-VIP

HT29-D4 cells grown as monolayers in 24-well multi-dish plates (2 cm²; 10⁶ cells/well) were washed twice with PBS containing 0.1% BSA and incubated in the binding medium with 0.5 μCi of mono[^{125}I]iodinated 4-AB-VIP (10⁻¹⁰ M) in the dark for 3 h at 13°C . Cell monolayers were then rinsed four times with BSA-free PBS and placed 10 cm under a u.v. lamp (Philips HP 3108) for 5 min.

Analysis of the photoaffinity-labelled proteins

After u.v. irradiation, cells were solubilized in 50 mM-Tris/HCl, pH 7.4, containing 1% Triton-X100, 5 mM-MgCl₂, 0.1% bacitracin, leupeptin (5 $\mu\text{g}/\text{ml}$), aprotinin (150 units/ml), 150 μM -phenylmethanesulphonyl fluoride and 2 mM-benzamidine for 20 min on ice. The whole mixture was then analysed by SDS/PAGE, as described

by Laemmli (1970), on a 12.5%-(w/v)-polyacrylamide slab gel under reducing conditions. Calibration proteins were: myosin (M_r 200 000), β -galactosidase (M_r 116 250), phosphorylase *b* (M_r 92 000), BSA (M_r 66 200) and ovalbumin (M_r 45 000). In some experiments, the gels were fixed, stained with Coomassie Brilliant Blue R250, dried, and then exposed to X-ray film (Fuji) for 3 weeks. In other experiments, radioactivity into the gel was quantified by cutting 3 mm-thick slices with an automatic gel slicer. Radioactivity contained in each slice was counted in a γ -radiation spectrometer (Kontron).

Endo F treatment of photoaffinity-labelled polypeptides

Endo F treatment was performed as previously described (El Battari *et al.*, 1987). Briefly, the photoaffinity-labelled cells were solubilized in 160 μ l of 0.1 M-sodium phosphate buffer, pH 6.1, containing 5 mM-EDTA, 5 mM-MgCl₂, 1% (v/v) 2-mercaptoethanol, 0.1% SDS and 1% Nonidet P40. The solubilized material was boiled for 2 min and allowed to cool at room temperature. A 20 μ l portion of Endo F [60 units/ml; provided in a 50% (v/v) glycerol/25 mM-EDTA solution] was then added. Incubation was carried out for 4 h at 37 °C. Samples were then analysed by SDS/PAGE.

RESULTS

Analytical data

Derivatization of native VIP. When native VIP was incubated with HSAB (molar ratio 1:1), analysis of the reaction mixture by RP-h.p.l.c. (Fig. 1a) revealed the presence of three major populations of derivatized VIP molecules (peaks 1, 2 and 3). These three species were eluted with increased elution time, well separated from native VIP. DMF by itself, at the same concentration [10% (v/v) final concn.], did not modify the elution profile of VIP (results not shown).

Analysis by RP-h.p.l.c. of fractions corresponding to peak 1, after u.v. irradiation, showed a decrease of 80% in the A_{280} of this species (Fig. 1c) compared with the same amount of non-irradiated material (Fig. 1b). It has been shown that u.v. irradiation of HSAB induces a loss of the 280 nm-absorbing property of this molecule, owing to the activation of the azido group (Hazum, 1983). Thus we concluded that peak 1 contained a u.v.-sensitive derivative. Similar data were obtained for peaks 2 and 3 (not shown).

Amino acid composition of 4-AB-VIP derivatives. The amino acid compositions of the compounds corresponding to peaks 1, 2 and 3 in Fig. 1(a) are given in Table 1. Each of them possesses an amino acid composition very similar to that expected for native VIP. This demonstrates that compounds 1, 2 and 3 are indeed VIP derivatives. Since the amide link between the ϵ -amino group of lysine residue and the azidobenzoate residue is labile under the hydrolysis conditions used, modification of lysine residues could not be detected. However, the more hydrophobic behaviour of compounds 1, 2 and 3 during RP-h.p.l.c. is direct evidence for a modification of VIP.

Binding of 4-AB-VIP to HT29-D4 cells. Competitive experiments between monoradioiodinated VIP and in-

Fig. 1. Analysis by RP-h.p.l.c. of 4-AB-VIP derivatives

(a) A 50 μ g portion of native VIP was derivatized in the presence of HSAB and analysed by RP-h.p.l.c. as described in the Experimental section. A_{280} (—) and the acetonitrile concentration (----) are plotted against elution time. The elution time of native VIP is indicated by an arrow at the top of the Figure. The filled-in peaks were also detected when HSAB hydrolysis products were injected alone on the column. Fractions corresponding to peak 1 were pooled and the acetonitrile evaporated off. (b) A part of this material was immediately re-analysed by RP-h.p.l.c., as in (a). (c) The same amount of material was u.v.-irradiated before RP-h.p.l.c.

creasing concentrations of RP-h.p.l.c.-purified native VIP or RP-h.p.l.c.-purified VIP derivatives (peaks 1, 2 and 3; Fig. 1a) have been performed on HT29-D4 cells. Competition curves are presented in Fig. 2. As already described for HT29 cells (Muller *et al.*, 1985a), the apparent K_D of native VIP for its receptor on HT29-D4 cells was 0.6 nM. The 4-AB-VIP derivatives were also able to displace ¹²⁵I-VIP with an apparent K_D of 2.5, 6.3 and 12.5 nM for peaks 1, 3 and 2 respectively. Therefore derivatization of VIP by HSAB induced a loss in affinity of the ligand for its receptor. However, this decrease in affinity was low enough to use the photosensitive VIP derivatives in binding experiments.

Derivatization of monoiodinated VIP. For unknown reasons, attempts to iodinate the VIP derivative from peak 1 resulted in a complete loss of the binding capacity of the derivative. Therefore radioiodination of VIP was performed before derivatization with HSAB.

Table 1. Amino acid analysis of the RP-h.p.l.c. fractions 1, 2 and 3 obtained after derivatization of VIP compared with that expected of the native VIP.

Amino acid	Composition (residues/molecule)			
	VIP	Peak 1	Peak 2	Peak 3
Asp	5	5.02	4.98	4.99
Thr	2	1.79	1.96	1.69
Ser	2	2.02	2.39	2.24
Glu	1	1.51	1.36	1.32
Ala	2	2.21	2.21	2.27
Val	2	1.58	1.64	1.69
Met	1	0.95	1.04	0.87
Ile	1	0.91	0.90	0.81
Leu	3	3.04	2.97	3.16
Tyr	2	1.77	1.71	1.41
Phe	1	0.62	0.90	1.06
His	1	1.03	1.09	1.15
Lys	3	2.74	2.94	2.65
Arg	2	1.61	2.02	1.89

Fig. 2. Competitive displacement of mono[¹²⁵I]iodinated VIP from HT29-D4 cells by native or 4-AB-VIP

For competitive-displacement experiments, after RP-h.p.l.c., the fractions corresponding to the different VIP derivatives (peaks 1, 2 and 3; Fig. 1a) were pooled and processed as described in the Experimental section for the purification of monoiodinated VIP. Cells (10^6) were incubated with 0.05 nM-¹²⁵I-VIP for 3 h at 13 °C in binding medium in the presence of increasing concentrations of native (□) or modified VIP from peak 1 (◆), 2 (◇) or 3 (■). Data are expressed as a percentage of tracer bound in the absence of unlabelled peptide. Each point is the mean of triplicates for one experiment representative of two. S.E.M. values are < 10 %.

Purification of mono[¹²⁵I]iodinated VIP was carried out by RP-h.p.l.c. A typical profile of the radioactivity eluted from the column is shown in Fig. 3(a). The fractions corresponding to peak A, characterized as mono[¹²⁵I]iodinated VIP (Martin *et al.*, 1986), were pooled and processed as described in the Experimental section.

A portion was derivatized in the presence of HSAB

Fig. 3. Purification of monoiodinated VIP by RP-h.p.l.c.: analytical RP-h.p.l.c. profile of 4-AB derivatives of monoiodinated VIP

(a) A 5 µg portion of native VIP was iodinated as described in the Experimental section. Purification of monoiodinated VIP was performed by RP-h.p.l.c., with a linear gradient of acetonitrile (25–40 % over 30 min). The radioactivity in each fraction (continuous line) and the acetonitrile concentration (broken line) are plotted against elution time. The elution time of native VIP is indicated by an arrow at the top of the Figure. Fractions corresponding to peak A were pooled and further processed as described in the Experimental section. (b) Purified monoiodinated VIP was derivatized with HSAB, and a portion (0.1 µCi) was analysed by RP-h.p.l.c., using a linear gradient of acetonitrile (10–50 % over 40 min). The profile of radioactivity eluted from the column (continuous line) and the acetonitrile concentration (broken line) are plotted against elution time. The arrow indicates the elution time of monoiodinated VIP from peak A in such a gradient of acetonitrile.

(molar ratio of reagent to reactive amino groups of monoiodinated VIP plus BSA 5:1) and subsequently analysed by RP-h.p.l.c. to demonstrate the presence of photoreactive derivatives, despite the presence of BSA in the reaction mixture. The profile of radioactivity eluted from the column is shown in Fig. 3(b). It was quite similar to the one obtained by derivatization of native VIP (Fig. 1a). Thus the three major species of VIP derivative were also detected under these experimental conditions.

Identification of the specifically photoaffinity-labelled VIP receptors on intact HT29-D4 cells

In routine experiments, monolayers of HT29-D4 cells were incubated in the presence of mono[¹²⁵I]iodinated

Fig. 4. Characterization of the photoaffinity-labelled polypeptides on HT29-D4 cells

Derivatization of monoiodinated VIP and incubation were carried out as described in the Experimental section. After u.v. irradiation, HT29-D4 cell monolayers were incubated with 1% Triton X-100. The whole solubilized material was then analysed by SDS/PAGE. (a) Autoradiography of a dried gel; (b) gel was sliced with an automatic gel slicer and the radioactivity of each slice counted. Incubations were made in the absence (a, lane A, and b, continuous line) or in the presence (a, lane B, and b, broken line) of $0.3 \mu\text{M}$ native VIP. (c) shows the profile obtained when the solubilized photoaffinity-labelled material was incubated in the absence (continuous line) or the presence (broken line) of Endo F at 37°C for 4 h before SDS/PAGE. M_r standards are indicated on the left of the autoradiogram and on the top of (b).

4-AB-VIP as described in the Experimental section. After u.v. irradiation, the photoaffinity-labelled cells were solubilized with 1% (v/v) Triton X-100 in the presence of MgCl_2 and proteinase inhibitors for 20 min on ice. The whole solubilization mixture was then subjected to SDS/PAGE under reducing conditions.

Autoradiography of the gel indicated the presence of a single photoaffinity-labelled polypeptide with a M_r of 70000 ± 5000 (s.d., $n = 13$ independent experiments) (Fig. 4a, lane A). Similar data were obtained when the gel was cut with an automatic gel slicer and the radioactivity of the slices counted (Fig. 4b).

The labelling of the M_r -70000 species was completely abolished when incubation was in the presence of $0.3 \mu\text{M}$ native VIP (Fig. 4a, lane B; Fig. 4b).

In order to demonstrate that the labelling of the M_r -70000 polypeptide was not due to the presence of BSA (M_r 66000) as carrier protein, we treated the cell lysate with Endo F. This led to a 20 kDa loss in the M_r of the photoaffinity-labelled polypeptide, much like that already described for the major M_r -67000 cross-linked polypeptide (El Battari *et al.*, 1987) (Fig. 4c).

Control experiments were performed either by using monoiodinated VIP instead of mono[^{125}I]iodinated 4-AB-VIP or by omitting the cell-monolayer u.v.-irradiation step (Figs. 5d and 5e respectively). In these two experiments no significant labelling of the M_r -70000 polypeptide was obtained.

The specificity of the photoaffinity-labelling reaction

has been investigated. On the one hand, incubation in the presence of $10 \mu\text{M}$ -glucagon never prevented the binding of VIP derivatives to the VIP receptor, and the M_r -70000 labelled polypeptide was fully recovered (Fig. 5b). On the other hand, incubation with $0.3 \mu\text{M}$ -VIP-(10–28)-peptide fragment, a concentration that displaces 25% of ^{125}I -VIP binding on HT29-D4 cells (Turner *et al.*, 1986), slightly decreased the extent of label (Fig. 5c). To further demonstrate the specificity of the labelling, the reaction was carried out on MDCK cells, which are devoid of VIP receptors (Muller *et al.*, 1985a). When these cells were used, no labelling of the M_r -70000 region of the gel was obtained (Fig. 5f).

DISCUSSION

The molecular characterization of the VIP receptor in various mammalian systems has been carried out almost exclusively by using the covalent-cross-linking approach [for a review of the different results obtained, see Marvaldi *et al.* (1986) and Luis (1987)]. The M_r of cross-linked polypeptides differ greatly from one system to another. These divergent results could reflect the following.

(i) Differential accessibility to the cross-linking reagents of components related to, or in the close vicinity of, the VIP-binding site. When high concentrations of hydrophobic homobifunctional reagents, such as dithio-bis(succinimidyl propionate) or disuccinimidyl suberate,

Fig. 5. Specificity of photoaffinity-labelling of VIP receptor: control experiments

(a), (b) and (c), Mono[125 I]iodinated 4-AB-VIP binding step was carried out in the absence (a) or the presence of either $10\text{ }\mu\text{M}$ -glucagon (b) or $0.3\text{ }\mu\text{M}$ -VIP-(10-28)-peptide fragment (c). After photolysis, the corresponding solubilized cell material was analysed by SDS/PAGE and the radioactivity was counted as described in Fig. 4. (d), (e) and (f), Radioactivity profiles obtained (d) when the experiment was performed with mono[125 I]iodinated VIP instead of mono[125 I]iodinated 4-AB-VIP, (e) when performed without the u.v.-irradiation step, and (f) when performed on MDCK-cell monolayers instead of HT29-D4 cells. For each experiment, the M_r -70000 region of the gel is indicated by an arrow.

are used, they could diffuse through the lipid bilayer of the plasma membrane and promote covalent links between VIP, VIP receptor and several other polypeptides, creating multimeric VIP labelled complexes. This could be the case for the VIP-binding site and the regulatory subunit of the adenylate cyclase. These two components have been shown to be very closely associated, since they are co-solubilized from rat liver plasma membranes and eluted together after gel-filtration chromatography (Couvineau *et al.*, 1986). The presence of the affinity-labelled M_r -120000 polypeptide on intact HT29 cells (Muller *et al.*, 1985a) could reflect such a phenomenon.

(ii) Proteolysis or deglycosylation when either crude or purified membranes were used instead of intact cells. The M_r -56000 cross-linked species, characterized in rat liver, was probably a proteolytic cleavage product of the M_r -80000 cross-linked polypeptide (Nguyen *et al.*, 1986). In the same way, the M_r -49000 cross-linked species, labelled in HT29 membranes (Marvaldi *et al.*, 1986), could result from proteolysis or deglycosylation of the major M_r -67000 cross-linked polypeptide characterized on intact cells.

In the present study we report the molecular identification of VIP receptor by a photoaffinity-labelling approach. We have synthesized mono[125 I]iodinated 4-AB-VIP derivatives that allowed us to label speci-

fically the VIP-binding site on intact HT29-D4 cells. Under our conditions of derivatization, 20–30 % of the mono[125 I]iodinated VIP molecules were modified. We assume that these 4-AB derivatives are fully potent with regard to binding to the VIP receptor, since their unlabelled analogues were able to compete efficiently with mono[125 I]iodinated VIP for the VIP-binding site.

After SDS/PAGE analysis of solubilized photoaffinity-labelled cells, a single polypeptide of M_r 70000 was detected. It is worth noting that, in all of the experiments that we have performed, the two minor cross-linked polypeptides of M_r 120000 and 34000 that were previously described (Muller *et al.*, 1985a) were not detected. The presence of only one photolabelled polypeptide suggested that VIP receptors on HT29-D4 cells contained a single species of binding-site subunit.

The M_r -70000 photoaffinity-labelled polypeptide behaved like the major M_r -67000 species characterized by covalent-cross-linking experiments on the same cells with the following criteria: (i) the label was completely abolished when the incubation step was performed in the presence of excess native VIP; (ii) the covalent linkage was not, or only slightly, affected when $10\text{ }\mu\text{M}$ -glucagon or $0.3\text{ }\mu\text{M}$ -VIP-(10-28)-peptide fragment respectively were used instead of native VIP; (iii) no label was obtained when MDCK cells were used instead of HT29-D4 cells; (iv) Endo F treatment of solubilized photo-

affinity-labelled cells resulted in a 20 kDa loss in the M_r of the cross-linked component.

Hence it seemed reasonable to consider that the M_r -67000 species characterized by cross-linking experiments and the photoaffinity-labelled M_r -70000 polypeptide were the same molecule. Therefore, assuming only one molecule of photoaffinity-cross-linked ^{125}I -VIP per polypeptide, the M_r of the VIP-binding site can be estimated at 67000 ± 5000 (s.d.) in the present system. However, we cannot explain why the M_r obtained by these two approaches were slightly different. Cross-linked polypeptides should be run side by side on the same polyacrylamide gel slab to assess any differences that could exist in the migrations of the two compounds. At the present time we think that the observed difference is not significant.

In numerous tissues, two classes of VIP-binding sites, one of high affinity and low capacity, the other of low affinity and high capacity, have been characterized by Scatchard analysis of results obtained from competitive displacement between native VIP and ^{125}I -VIP. Nevertheless, in a recent paper, Robichon & Marie (1987) have indicated that a single binding subunit was detected by photoaffinity labelling of VIP receptors on rat liver plasma membrane, although two classes of binding sites have been described in this system. Similar data have also been obtained with the glucagon receptor (Iyengar & Herberg, 1984). This suggests that the characterization of two classes of binding sites does not necessarily imply two physically distinct receptor molecules. These differences could result, for example, from differential coupling with the adenylate cyclase system.

It has been demonstrated that VIP was internalized by HT29 cells (Muller *et al.*, 1985b; Marie *et al.*, 1986). Furthermore, incubation with native VIP results in a loss in VIP-binding capacity of these cells (Luis *et al.*, 1986), suggesting strongly that VIP enters the cell by receptor-mediated endocytosis. The photoaffinity probes we have synthesized allowed us to tag the VIP receptors, keeping (probably) intact the plasma-membrane dynamic capacity. They could be useful tools to demonstrate the presence of VIP receptor(s) in different intracellular compartments and to describe the definite traffic (internalization and recycling) of the VIP-receptor complexes within the cell.

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