Solubilization and Hydrodynamic Characterization of Guanine Nucleotide Sensitive Vasoactive Intestinal Peptide–Receptor Complexes from Rat Intestine†

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ABSTRACT: The purpose of this work was to solubilize vasoactive intestinal peptide (VIP) receptors from rat small intestinal plasma membranes and to analyze the nature and function of its molecular form(s) in a nondenaturing environment. Membranes were incubated with 3 nM ^{125}I-VIP, washed, and treated with 1% Triton X-100. Chromatography on Sephadex G-50 showed that 60% of the extractable radioactivity was eluted with macromolecular components in the void volume. This radioactive material was dramatically reduced when 1 μM unlabeled VIP was present in the incubation medium or when membranes were pretreated with trypsin or dithiothreitol. Macromolecular components that had bound ^{125}I-VIP were further chromatographed on Sephacryl S-300. Two peaks were observed: a major one (80%) and a minor one (20%) with Stokes radii of 5.2 and 3.1 nm, respectively. The labeling of both components was inhibited by unlabeled VIP or peptide with NH_2-terminal histidine and COOH-terminal isoleucine amide (a VIP agonist). The presence of GTP (0.1 mM) in the incubation medium of membranes completely abolished the labeling of the 5.2-nm component but did not affect that of the 3.1-nm one. Moreover, GTP induced dissociation of ^{125}I-VIP from the 5.2-nm component isolated by Sephacryl S-300 chromatography. This effect was time dependent and nucleotide specific. In contrast, GTP did not affect the stability of the 3.1-nm component. After cholera toxin catalyzed [^{32}P]ADP-ribosylation of membranes, chromatography of solubilized material on Sephacryl S-300 showed that a peak of ^{32}P radioactivity was coeluted with the 5.2-nm component. SDS–PAGE analysis revealed the presence in this peak of the M_r 42000 α subunit of the Gs protein. Sucrose density gradient ultracentrifugation of the 5.2- and 3.1-nm components isolated on Sephacryl S-300 showed that they displayed apparent sedimentation coefficients of 6.7 and 3.9 S, respectively. From these results, the molecular weight of both components was estimated to be 152000 and 54000, respectively. It was concluded that intestinal VIP–receptor complexes of M_r 54000 were solubilized in a major form of M_r 152000 containing a Gs protein that was sensitive to GTP regulation.

Intestinal VIP[1] receptors (Prieto et al., 1979) translate the peptide–receptor interaction into secretion of water and electrolytes (Laburthe & Dupont, 1982) through a cascade of events initiated by mobilization of the stimulatory GTP regulatory protein Gs and activation of adenylate cyclase (Amiranoff et al., 1978), followed by elevation of cellular cAMP levels (Laburthe et al., 1979a), activation of cAMP-dependent protein kinase (Laburthe et al., 1979b), and phosphorylation of proteins (Cohn, 1987). In the rat small intestinal epithelium, VIP receptors have undergone extensive functional and pharmacological characterization (Laburthe & Dupont, 1982]. Also, preliminary studies have investigated their molecular properties by means of covalent cross-linking techniques presenting evidence for a high-affinity ligand binding unit of 73000 daltons (Laburthe et al., 1984). Although cross-linking is a fruitful preliminary approach for studying structure of receptor proteins (Ji, 1979) and, under some circumstances, their interaction with other membrane proteins (Ji, 1979; Couvineau et al., 1986), this technique suffers some limitations due to the low yield of cross-linking usually obtained, the fact that covalent ligand–receptor complexes are physiologically irrelevant, and the possibility of uncontrolled cross-linking to neighboring unknown proteins.

In order to circumvent these difficulties and with the aim to document the hydrodynamic properties of intestinal VIP receptors in a nondenaturing environment, we have solubilized VIP–receptor complexes from rat small intestine using Triton X-100 under conditions previously developed for studying rat liver receptors (Couvineau et al., 1986). We provide evidence for the solubilization of a major M_r 152000 component that is sensitive to GTP regulation and likely represents the VIP receptor associated with the Gs protein. Since cross-linking experiments (Couvineau & Laburthe, 1985a) and functional studies (Robberecht et al., 1982) previously suggested heterogeneity of VIP receptors among tissues, the results obtained in rat intestine will be compared to those recently reported in rat liver (Couvineau et al., 1986) and guinea pig lung (Paul & Said, 1987).

Materials and Methods

Materials. Purified pig VIP was provided by Professor V. Mutt (Karolinska Institute, Stockholm, Sweden) and was radioiodinated with ^{125}I by the chloramine T method at a specific activity of 750 Ci/mmol as described (Laburthe et al., 1977). It displayed the same activity as native VIP in stimulating cAMP production in the cultured cell line HT-29

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[1] Abbreviations: Gs, the guanine nucleotide regulatory protein that mediates hormonal stimulation of adenylate cyclase; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; VIP, vasoactive intestinal peptide.

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(Laburthe et al., 1978). Synthetic peptide with NH_2-terminal histidine and COOH-terminal isoleucine amide was a gift from Professor L. Moroder (Max Planck Institute, Martinsried, Federal Republic of Germany). Trypsin from bovine pancreas (type III-S), cholera toxin, Triton X-100, nucleotides, dithiothreitol, Hepes, bacitracin, and calibrating proteins for hydrodynamic measurements were purchased from Sigma. Chemicals and unstained protein markers for SDS gel electrophoresis were from Bethesda Research Laboratories and Sephadex G-50 and Sephacryl S-300 from Pharmacia. [α-^{32}P]NAD was from New England Nuclear and D_2O from Commissariat ã l'Energie Atomique, Gif-sur-Yvette, France.

Preparation of Intestinal Epithelial Plasma Membranes. Two-month-old male Wistar rats fed ad libitum were used at 0900–1000 h. After decapitation, the jejuno-ileum was removed, and isolated epithelial cells were obtained on ice as described (Prieto et al., 1979). Plasma membranes were then prepared immediately from isolated cells as described (Laburthe et al., 1986). The plasma membrane enriched fraction was kept at −80 °C until use. No loss of VIP receptors was observed for up to 4 months.

Binding of ^{125}I-VIP to Membranes. Binding of ^{125}I-VIP to membranes was examined as described (Laburthe et al., 1984) in a total volume of 5 mL of 20 mM Hepes buffer, pH 7.5, containing 1.6% (w/v) bovine serum albumin and 0.08% (w/v) bacitracin. Membranes at 500 μg of protein/mL were incubated for 30 min at 30 °C with 3 nM ^{125}I-VIP and other compounds when necessary. The incubation was stopped by adding 35 mL of ice-cold 20 mM Hepes buffer, pH 7.5, followed by centrifugation for 10 min at 30000*g*. The membrane pellet containing bound ^{125}I-VIP was used immediately for subsequent solubilization of membrane proteins. Specific binding calculated as the difference between the amount of ^{125}I-VIP bound in the absence (total binding) and in the presence (nonsaturable binding) of 1 μM unlabeled VIP represented 65–70% of total binding.

Solubilization of Membrane Proteins. The membrane pellet obtained after incubation with ^{125}I-VIP (see above) was incubated for 30 min in ice at a concentration of 500 μg of protein/mL in 20 mM Hepes buffer, pH 7.5, containing 1% (w/v) Triton X-100. The solubilized proteins were separated from the nonextractable material by centrifugation for 30 min at 40000*g* or for 60 min at 100000*g*. Seventy percent of the radioactivity initially bound to membranes and 50% of membrane proteins were recovered in the supernatant whatever the conditions of centrifugation.

Gel Filtrations. ^{125}I-VIP associated with macromolecular components was separated by gel filtration from free ^{125}I-VIP that may occur during solubilization of membrane proteins or is generated during dissociation experiments. For that purpose, a Sephadex G-50 (superfine) column (0.9 × 30 cm) was used. It was equilibrated and eluted at 4 °C at a flow rate of 1 mL min^{-1} with 20 mM Hepes buffer, pH 7.5, containing 50 mM KCl, 0.1% (v/v) Triton X-100, and 1% (w/v) bovine serum albumin. Fractions of 500 μL were collected.

In order to determine the Stokes radius of the macromolecular components associated with ^{125}I-VIP, fractions eluted in the void volume of the above-described Sephadex G-50 column were pooled and submitted to gel filtration on a Sephacryl S-300 column (60 × 1 cm) in tandem with a Sephadex G-50 column (30 × 1 cm). Columns were equilibrated and eluted at 4 °C with the above-mentioned buffer. Fractions of 1 mL were collected. Blue dextran 2000 and $K_3Fe(SCN)_6$ were used to determine and void volume (V_0) and the total liquid volume (V_t) of the columns, respectively. Columns were calibrated with marker proteins of known Stokes radius as shown in the legends to figures.

Sucrose Density Gradient Ultracentrifugation. Apparent sedimentation coefficients of VIP–receptor complexes were determined by ultracentrifugation of peaks obtained by gel filtration on Sephacryl S-300 (see above). Linear 5–20% gradients (4 mL) made in H_2O or D_2O contained 25 mM Hepes, pH 7.5, 50 mM KCl, and 0.1% (v/v) Triton X-100. Centrifugation was performed at 4 °C for 16 h at 43 000 or 55 000 rpm for H_2O or D_2O sucrose gradients, respectively. Calibration curves for the determination of sedimentation coefficients were constructed according to the method of Martin and Ames (1961) as described previously (Couvineau et al., 1986) using the protein markers of known sedimentation coefficient listed in the legend to Figure 6.

Treatment of Membranes with Trypsin or Dithiothreitol. Membranes (0.5 mg of protein/mL) were incubated with trypsin (0.5 mg/mL) for 30 min at 37 °C in 20 mM Hepes buffer, pH 7.5. At the end of the incubation, N^{α}-*p*-Tosyl-L-lysine chloromethyl ketone (1 mg/mL) was added, and the membrane pellet was recovered by centrifugation for 10 min at 30000*g*. Treatment of membranes (0.5 mg/mL) with 20 mM dithiothreitol was performed as described previously (Couvineau & Laburthe, 1985b).

Cholera Toxin Catalyzed ADP-Ribosylation of Membranes. Activated (Cassel & Selinger, 1977; Couvineau et al., 1986) cholera toxin (50 μg/mL) was incubated with intestinal membranes (500 μg of protein/mL) for 30 min at 30 °C with 5 μM [^{32}P]NAD as described (Couvineau et al., 1986). After solubilization, membrane proteins were subjected to gel permeation chromatography (see above) or SDS–polyacrylamide gel electrophoresis as described elsewhere (Couvineau et al., 1986).

Protein Determination. Proteins were determined by the method of Bradford (1976) with bovine serum albumin as the standard. When Triton X-100 was present, its concentration was 0.01% (v/v) and it did not interfere with the protein estimation.

Results

Rat intestinal plasma membranes were incubated with 3 nM ^{125}I-VIP, washed free of unbound VIP, and treated with 1% Triton X-100. Chromatography of the solubilized material on Sephadex G-50 showed that 60% of the radioactivity was eluted with macromolecular components in the void volume of the column (Figure 1). The remaining radioactivity was coeluted with free ^{125}I-VIP, indicating partial dissociation of ^{125}I-VIP–receptor complexes during solubilization of membranes with Triton. As tested by rechromatography on Sephadex G-50, the radioactive material eluted in the void volume remained stable for at least 4 h at 4 °C, whereas significant dissociation was observed on storage at room temperature; e.g., 30% of the radioactivity initially found in the void volume was eluted with free ^{125}I-VIP after 4 h (not shown). Therefore, all subsequent hydrodynamic characterizations were performed at 4 °C. Several lines of evidence indicated that the radioactive material eluted in the void volume contained ^{125}I-VIP–receptor protein complexes. First, when 1 μM unlabeled VIP was added together with ^{125}I-VIP in the incubation medium of membranes, an important decrease of the radioactivity eluted in the void volume was observed (Figure 1). Second, pretreatment of membranes with trypsin (0.5 mg/mL) for 1 h at 37 °C followed by incubation with ^{125}I-VIP and solubilization (see Materials and Methods) resulted in an almost complete absence of radioactivity in the void volume of the column (not shown), in accordance with

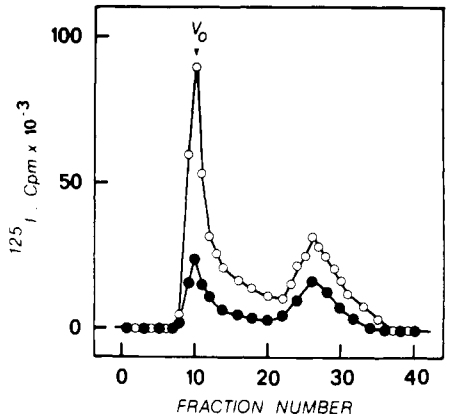

FIGURE 1: Chromatographic profile of solubilized membranes labeled with ^{125}I-VIP on Sephadex G-50. Intestinal membranes were incubated with ^{125}I-VIP in the absence (○) or in the presence (●) of 1 μM unlabeled VIP, washed and solubilized as described under Materials and Methods. The solubilized extract was then applied on a Sephadex G-50 column. V_0 indicates the void volume of the column.

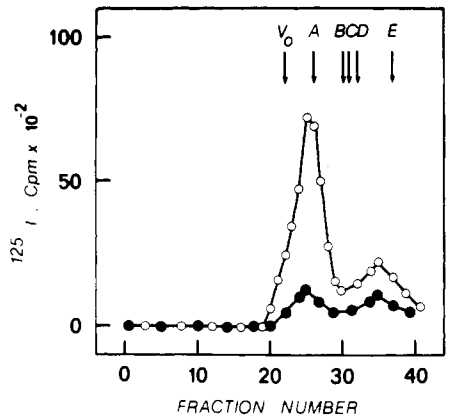

FIGURE 2: Gel filtration profile of the solubilized ^{125}I-VIP–receptor complexes on Sephacryl S-300. Fractions eluted in the void volume of the Sephadex G-50 column (see Figure 1) were pooled and applied on a Sephacryl S-300 column as described under Materials and Methods. The symbols have the same meaning as in Figure 1. The column was calibrated with marker proteins of known Stokes radius: (A) gammaglobulin (5.1 nm); (B) bovine serum albumin (3.55 nm); (C) hemoglobin (3.2 nm); (D) ovalbumin (3.05 nm); (E) myoglobin (1.9 nm). V_0 indicates the void volume of the colunm.

the sensitivity of intestinal VIP receptors to trypsin attack (Sarrieau et al., 1983). Third, similar pretreatment with dithiothreitol (see Materials and Methods) also markedly reduced the radioactivity in the void volume (not shown), in agreement with the known sensitivity of VIP receptor binding activity to reducing agents (Robberecht et al., 1984; Couvineau & Laburthe, 1985b).

Further analysis of macromolecular components that had bound ^{125}I-VIP was obtained by submitting the material present in the void volume of the Sephadex G-50 column to chromatography on Sephacryl S-300. Two peaks were observed: a major one (80% of the radioactivity) eluted in fraction 25, and a minor one (20% of the radioactivity) eluted in fraction 35 (Figure 2). Fractionation of calibrating proteins of known Stokes radii on the column (Figure 2) made it possible to estimate the Stokes radius of each component, e.g., 5.2 and 3.1 nm for the major and minor components, respectively. Both components appeared to be labeled specifically by ^{125}I-VIP. Indeed, addition of 1 μM unlabeled VIP during the initial incubation of membranes with ^{125}I-VIP reduced the labeling of the major and minor components by 80% and 60%, respectively (Figure 2). Futhermore, peptide with NH_2-terminal histidine and COOH-terminal isoleucine amide, which

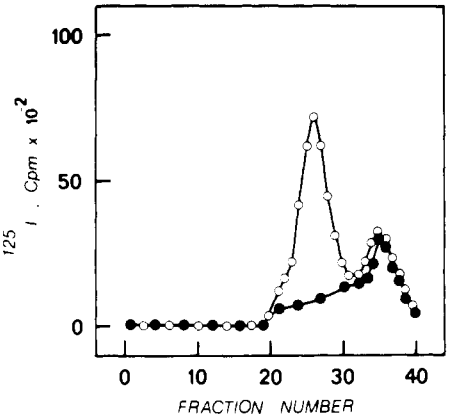

FIGURE 3: Sephacryl S-300 chromatographic profile of solubilized ^{125}I-VIP–receptor complexes after incubation of membranes with GTP. Conditions were as described in the legend to Figure 2. Elution profiles correspond to membranes incubated with ^{125}I-VIP in the absence (○) and in the presence (●) of 0.1 mM GTP.

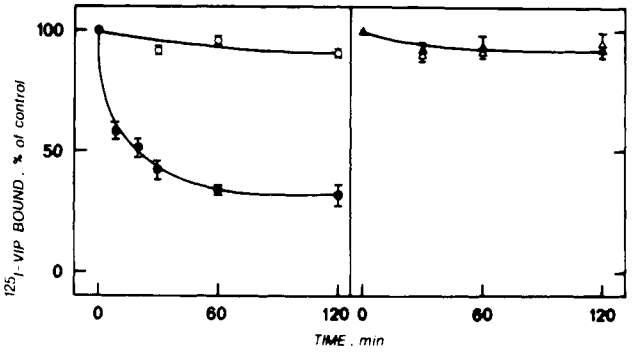

FIGURE 4: Effect of GTP on the dissociation of solubilized ^{125}I-VIP–receptor complexes. The 5.2- and 3.1-nm components were isolated on Sephacryl S-300 as shown in Figure 2. Dissociation was initiated by adding to the 5.2-nm component (left) or 3.1-nm component (right) 1 μM unlabeled VIP without (open symbols) or with (filled symbols) 0.1 mM GTP. After incubation at 30 °C for the indicated times, the radioactivity remaining bound to the macromolecular components was determined by chromatography on Sephadex G-50, which separates macromolecular components from free ^{125}I-VIP (see Figure 1). Results represent the mean ± SE of three experiments.

is a VIP agonist in various tissues (Bataille et al., 1980), reduced the labeling of the two components when added at a concentration of 10 nM together with ^{125}I-VIP (not shown).

In order to investigate the nature and function of the 5.2- and 3.1-nm components, we tested the effect of GTP, added in the incubation medium of membranes, on their labeling. In the presence of GTP, the labeling of the 5.2-nm component was dramatically decreased, whereas the labeling of the 3.1-nm component was essentially unaffected (Figure 3). This observation suggested the presence of a GTP-regulatory protein functionally associated with the VIP receptor in the 5.2-nm component. This hypothesis was further tested by studying the effect of GTP on the dissociation of ^{125}I-VIP from the 5.2- and 3.1-nm components isolated by chromatography on Sephacryl S-300. These components were incubated with 0.1 mM GTP and then chromatographed on a Sephadex G-50 column to determine free ^{125}I-VIP. It appeared that GTP induced a time-dependent dissociation of ^{125}I-VIP from the 5.2-nm component (Figure 4, left). About 70% of the radioactivity was detected as free ^{125}I-VIP after 60 min of incubation at 30 °C (Figure 4, left). In contrast, the 3.1-nm component was insensitive to GTP regulation (Figure 4, right). These results strongly suggested that the functional interaction between the VIP receptor and GTP-regulatory protein survives

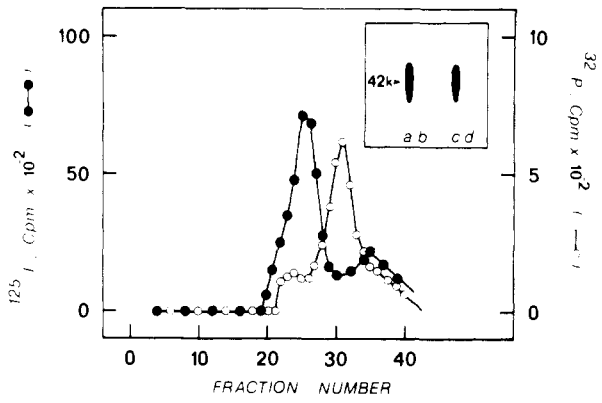

FIGURE 5: Chromatography on Sephacryl S-300 of solubilized membrane proteins [^{32}P]ADP-ribosylated by cholera toxin. Membranes were incubated in the presence of [^{32}P]NAD with activated cholera toxin, solubilized, and chromatographed on Sephacryl S-300 as described under Materials and Methods. The ^{32}P radioactivity eluted (○) was determined by scintillation counting. In parallel experiments, membranes were incubated with ^{125}I-VIP, solubilized, and chromatographed on the same Sephacryl S-300 column, and the elution profile (●) is of ^{125}I-VIP bound to macromolecular components. (Inset) SDS–PAGE analysis of [^{32}P]ADP-ribosylated proteins coeluted with the 5.2-nm component of VIP binding and from intestinal membranes. Membranes were incubated in the presence of [^{32}P]NAD with (lanes a and c) or without (lanes b and d) activated cholera toxin. [^{32}P]ADP-ribosylated proteins were submitted to an SDS–PAGE analysis on a 10% polyacrylamide slab gel. In lanes a and b, [^{32}P]ADP-ribosylated membrane proteins were resolved on SDS–PAGE after solubilization with SDS. In lanes c and d, [^{32}P]ADP-ribosylated membranes were solubilized with Triton X-100 and chromatographed on a Sephacryl S-300 column (see main panel). The fractions eluted in the peak of ^{32}P radioactivity coeluted with the 5.2-nm component of VIP binding (see main panel) were pooled and precipitated with 10% trichloracetic acid. After centrifugation, the pelleted material was solubilized with SDS and submitted to SDS–PAGE.

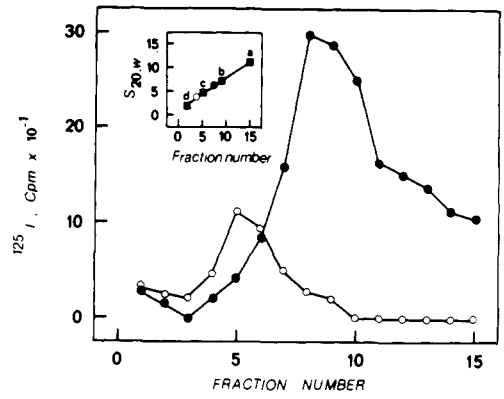

FIGURE 6: Sucrose density gradient ultracentrifugation of ^{125}I-VIP–receptor complexes solubilized from rat intestinal membranes. The 5.2-nm (●) and 3.1-nm (○) components isolated by chromatography on Sephacryl S-300 (see Figure 2) were centrifuged separately through a 5–20% sucrose gradient in H_2O as described under Materials and Methods. (Inset) $s_{20,w}$ values of known protein markers are plotted as a function of travel (fraction number). The $s_{20,w}$ values of ^{125}I-VIP–receptor complexes were estimated from this standard curve. The following markers were used: (a) catalase from bovine liver (11.45 S); (b) bovine γ-globulin (7.2 S); (c) bovine serum albumin (4.6 S); (d) cytochrome *c* (1.71 S).

solubilization of membrane proteins with Triton X-100. The nucleotide specificity of the effect was tested by incubating the 5.2-nm component with GTP, ATP, UTP, and CTP at a concentration of 0.1 mM for 60 min at 30 °C. Only GTP was able to induce the dissociation of ^{125}I-VIP from the 5.2-nm component.

Since VIP is a potent stimulator of intestinal adenylate cyclase activity (Amiranoff et al., 1978), the stimulatory GTP-regulatory protein Gs was likely to be the G protein associated with the VIP receptor in the solubilized 5.2-nm component. To investigate this hypothesis and to further argue for a physical interaction between VIP receptor and the G protein, intestinal membranes were treated with cholera toxin in the presence of [^{32}P]NAD under conditions in which the Gs protein is specifically labeled by ADP-ribosylation of its α subunit (Johnson et al., 1978; Northup et al., 1980; Scheifer et al., 1982). After chromatography of Triton X-100 solubilized membrane proteins on Sephacryl S-300, two peaks of ^{32}P radioactivity were observed (Figure 5). A major peak was eluted in fraction 31, where the free Gs protein was expected to be found. However, a minor peak of ^{32}P radioactivity was coeluted in fraction 25 with the 5.2-nm component specifically labeled by ^{125}I-VIP. This observation was consistent with the association of part of the Gs proteins population with the VIP receptor. The [^{32}P]ADP-ribosylated membrane proteins were further analyzed by SDS–PAGE (Figure 5, inset). The ^{32}P radioactivity was associated with a protein of M_r 42 000 (Figure 5, inset), in agreement with the fact that cholera toxin stimulation of ADP-ribosylation of the Gs protein occurs on its α subunit of M_r 42 000 (Johnson et al., 1978; Schleifer et al., 1982). The peak fraction of [^{32}P]ADP-ribosylated protein that coeluted with the 5.2-nm component of VIP binding was also analyzed with SDS–PAGE, showing the presence of the α subunit of the Gs protein (Figure 5, inset). In contrast, no peak of ^{32}P radioactivity was coeluted with the 3.2-nm component of VIP binding on Sephacryl S-300 (Figure 5). Therefore, these data tend to suggest that the 5.2-nm component contains the VIP receptor associated with a Gs protein, whereas the 3.1-nm component could be the VIP receptor alone.

To substantiate this assumption, the hydrodynamic properties of both components were further analyzed by sucrose density gradient ultracentrifugation to provide a good estimate of their molecular weight. For that purpose the two peaks eluted from the Sephacryl S-300 column (see Figure 2) were subjected to ultracentrifugation on a linear 5–20% sucrose gradient made up in H_2O and calibrated with marker proteins of known sedimentation coefficient (Figure 6). The major component, eluted from the Sephacryl S-300 column with a Stokes radius of 5.2 nm, migrated with an apparent sedimentation coefficient ($s_{20,w}$) of 6.7 S. The minor component of Stokes radius of 3.1 nm migrated with an apparent $s_{20,w}$ of 3.9 S. Both components migrated as single peaks on sucrose gradient, showing that ultracentrifugation detected no further heterogeneity in VIP binding species. The apparent sedimentation coefficients determined in D_2O (not shown) were the same as those calculated from gradients made up in H_2O, supporting the idea that two components and the calibrating proteins have a very similar partial specific volume. Therefore, the average partial specific volume of calibrating proteins (0.74 mL/mg) was used for calculating their molecular weight. The frictional ratio and molecular weights of the two components calculated from gel filtration (Figure 2) and ultracentrifugation (Figure 6) experiments are reported in Table I. It is of interest to notice that the difference between the molecular weight of the major component (M_r 152 000) and that of the minor one (M_r 54 000) compares well with the M_r of the Gs protein, i.e., 90 000.

Discussion

This paper documents the hydrodynamic properties of VIP receptors in the small intestine, which represents a most important target tissue for this neuropeptide (Laburthe & Du-

Table I: Hydrodynamic Parameters of VIP–Receptor Complexes Solubilized from Rat Small Intestine

parameter	major component	minor component
Stokes radius, a (nm)	5.2 ± 0.1 (25)[a]	2.1 ± 0.1 (22)[a]
sedimentation coeff, $s_{20,w}$ (S)	6.7 ± 0.2 (5)[a]	3.9 ± 0.1 (5)[a]
M_r[b]	152 000 ± 3000	54 000 ± 2000
	145 000–161 000[d]	47 000–57 000[d]
frictional ratio, f/f_0[c]	1.30	1.09

[a] Values are means ± SE of number determinations indicated in parentheses. [b] Calculated from $M_r = [6\pi N\eta_{20,w}/(1 - \bar{v}\rho_{20,w})]as_{20,w}$, where N is Avogadro's number, $\eta^{20,w}$ the viscosity of water at 20 °C [0.01002 g/(cm·s)], and $\rho_{20,w}$ the density of water at 20 °C (0.99823 g/mL). The partial specific volume of VIP–receptor complexes (v) is assumed to be identical with that of the standard proteins, e.g., 0.74 mL/mg. [c] The frictional ratio was calculated by assuming a hydration of 0.2 g/g of proteins as follows: $f/f_0 = a[4\pi N/3M_r(\bar{v} + 0.2)]^{1/3}$. [d] Range values calculated by using 1 SE above and below each parameter.

pont, 1982). Two forms of VIP–receptor complexes differing by their molecular size and functional properties are solubilized from rat intestinal membranes by Triton X-100. Gel filtration and sucrose density gradient ultracentrifugation indicate the solubilization of a major form (80%) and a minor one (20%) with Stokes radii of 5.2 and 3.1 nm and sedimentation coefficients of 6.7 and 3.9 S, respectively. From these values, the M_r of each form has been estimated to be 152 000 and 54 000, respectively. Since no significant difference is observed between sedimentation coefficient values measured in H_2O and D_2O, it has been assumed that the partial specific volumes of both forms are similar to that of the globular protein markers (Clarke, 1975). In this context, it may be suggested that both forms bind a small amount of Triton X-100, but because of the identical sedimentation coefficients measured in H_2O and D_2O, this amount cannot be estimated (Clarke, 1975). With respect to the functional significance of the two macromolecular components that have bound VIP, it may be stressed that they are specific since their labeling is dramatically reduced when unlabeled VIP is present in the initial incubation medium of membranes with ^{125}I-VIP. Their peptide specificity is also supported by the ability of peptide with NH_2-terminal histidine and COOH-terminal isoleucine amide, a VIP agonist in various tissues including intestine (Bataille et al., 1980), to decrease their labeling. However, the two forms of VIP–receptor complexes differ by their sensitivity to GTP regulation. Indeed, ^{125}I-VIP dissociates from the major M_r 152 000 component when it is incubated in the presence of GTP, whereas the minor M_r 54 000 component is insensitive to GTP regulation (see Figure 4). This observation fits well with the fact that when membranes are initially incubated with ^{125}I-VIP in the presence of GTP, subsequent solubilization of membranes reveals that the labeling of the major component is almost completely abolished, whereas the minor component is unaffected (see Figure 3). The sensitivity of the M_r 152 000 component to GTP regulation in solution is in consonance with the regulation of membrane-bound VIP receptors by GTP, which inhibits VIP binding to intestinal membranes by enhancing the rate of dissociation of VIP from its receptors (Amiranoff et al., 1980a). Moreover, it may be noted that the nucleotide specificity of the effect is identical for the dissociation of ^{125}I-VIP from the solubilized M_r 152 000 component and for the inhibition of ^{125}I-VIP binding to membranes (Amiranoff et al., 1980a). Previous observations showing the regulation of VIP binding to intestinal receptors by GTP (Amiranoff et al., 1980a) and the mandatory role of GTP for stimulation of adenylate cyclase by VIP (Amiranoff et al., 1980b) supported the functional coupling of intestinal VIP receptors with a stimulatory guanine nucleotide regulatory protein Gs. The present data indicating that GTP sensitivity of VIP receptors survives solubilization support a major proportion of VIP receptors population being closely coupled with the Gs protein in the plasma membrane of enterocytes. Moreover, they strongly suggest physical association of VIP receptors with the Gs protein in the M_r 152 000 component. This is further supported by experiments in which cholera toxin is used to catalyze the ADP-ribosylation of the Gs protein. Indeed, it appears clearly that a significant proportion of the [^{32}P]ADP-ribosylated Gs proteins is coeluted with the ^{125}I-VIP-labeled M_r 152 000 component on Sephacryl S-300 (see Figure 5). Taken together, these data support the idea that the M_r 152 000 component is a ternary complex consisting of VIP, the receptor, and the Gs protein. Considering the M_r of the Gs protein, i.e., about 90 000 (Gilman, 1987), the M_r of the VIP receptor itself can be roughly estimated to be 60 000. This compares well the estimated M_r of the minor form that is insensitive to GTP regulation, i.e., 54 000 (see Table I), suggesting that it represents the VIP–receptor complex uncoupled with the Gs protein. This M_r estimated here from hydrodynamic parameters of Triton X-100 solubilized membranes protein is slightly different from the M_r of the VIP receptor previously determined by using the covalent cross-linking technique in rat small intestine, i.e., 73 000 (Laburthe et al., 1984). The reason the two techniques give different results is unclear at the present time but may be tentatively ascribed to various causes: (1) Some small errors may be associated with the determination of hydrodynamic parameters of solubilized membrane proteins (Tanford & Reynold, 1976). (2) Covalent labeling of receptors using chemical cross-linker may result in the cross-linking not only of ligand with receptors but also of receptors with neighboring proteins in the plasma membrane. It may be stressed, however, that cross-linking of ^{125}I-VIP to intestinal membranes using a very low concentration (0.1 mM) of disuccinimydyl suberate does not modify the pattern of protein labeling as seen by SDS–PAGE analysis.[2] Nor was there any modification of this pattern when cross-linking was performed at 0 °C, which slows the diffusion of proteins in the plane of plasma membrane of intestinal epithelial cells (Zomiek et al., 1980) and prevents the detection of cross-linked complexes due to random collision (Peters & Richards, 1977). Therfore, cross-linking of intestinal VIP receptors to a neighboring protein, if any, would more likely occur with a closely associated protein. In that respect, subunits of the Gs protein are good candidates. In previous cross-linking experiments performed in rat intestine, SDS–PAGE analysis of membrane proteins also showed a minor M_r 33 000 low-affinity VIP binding component, the biological significance of which remained conjectural (Laburthe et al., 1984). For unknown reasons, this component is not observed in the Triton-solubilized material. This may be related to the different effectiveness of Triton and SDS for solubilizing membrane proteins. Alternatively, this component may have been missed in the present study because its labeling is very low and the Sephacryl S-300 column does not exhibit sufficient resolutive characteristics.

Heterogeneity of VIP receptors among tissues has been previously suggested on the basis of pharmacological properties (Robberecht et al., 1986), efficacy of VIP in stimulating adenylate cyclase activity [reviewed in Couvineau and Laburthe (1985a)], and molecular weight of ligand binding units determined by chemical cross-linking (Couvineau & Laburthe, 1985a). It is therefore of interest to compare the properties

[2] A. Couvineau and M. Laburthe, unpublished data.

of VIP receptors solubilized from rat intestine (this paper) with those described in the two other tissues documented so far, i.e., rat liver (Couvineau et al., 1986) and guinea pig lung (Paul & Said, 1987). The overall similarities are the following: (1) Two components of VIP binding differing by their molecular size and sensitivity to GTP regulation have been observed. (2) The major high-affinity receptor component is sensitive to GTP regulation, suggesting tight association with a GTP-regulatory protein. Since lung VIP receptors have not been analyzed by sucrose density gradient ultracentrifugation (Paul & Said, 1987), their hydrodynamic properties cannot be further compared with those of rat intestinal receptors. Moreover, lung receptor (Paul & Said, 1987) originated from a different species and was solubilized with CHAPS instead of Triton X-100. Fortunately, VIP receptors from rat liver (Couvineau et al., 1986) and rat small intestine can be more readily compared since they have been solubilized by use of the same methodology. In the two tissues, the minor components, which likely represent the VIP receptor uncoupled with the GTP-regulatory protein, have very similar hydrodynamic properties including Stokes radius, sedimentation coefficient, M_r, and frictional ratio [see Couvineau et al. (1986) and Table I]. With respect to the major components of VIP binding that are sensitive to GTP regulation, it appears that they display identical M_r in rat liver (Couvineau et al., 1986) and rat small intestine (see Table I). However, they differ by some hydrodynamic properties, e.g., Stokes radius, 5.2 vs 5.8 nm; sedimentation coefficient, 6.7 vs 6.0 S; and frictional ratio, 1.30 vs 1.52 in rat small intestine and rat liver (Couvineau et al., 1986), respectively. Although we cannot exclude some errors in the determination of hydrodynamic parameters of solubilized membrane receptors (Tanford & Reynold, 1976), these results suggest a different spatial interaction between VIP receptors and GTP-binding proteins in liver and intestine, resulting in a more spherical complex in intestine. This may favor the hypothesis of cross-linking of VIP receptors to a subunit of the GTP-regulatory protein in intestine (see above) and can be correlated with the much lower efficacy of VIP in stimulating adenylate cyclase activity in liver compared to intestine (Couvineau & Laburthe, 1985b).

In summary, the findings reported here provide strong evidence that rat intestinal VIP receptors are solubilized in a major macromolecular form of M_r 152 000 consisting of VIP, the receptor, and the GTP-regulatory protein Gs. In addition to providing biochemical evidence for the role of Gs in the mechanism of VIP action in intestine, these results may allow for the more complete molecular characterization of VIP-mediated events coupled to the stimulation of adenylate cyclase.

Registry No. VIP, 37221-79-7; GTP, 86-01-1.

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