Vasoactive Intestinal Peptide Receptor on Liver Plasma Membranes: Characterization as a Glycoprotein†

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ABSTRACT: The receptor for vasoactive intestinal peptide (VIP) was identified in rat liver plasma membranes after covalent cross-linking to ^{125}I-VIP by three different agents [disuccinimido dithiobis(propionate), disuccinimido suberate, and succinimido 4-azidobenzoate] and examined by sodium dodecyl sulfate–acrylamide electrophoresis. Regardless of the presence of reducing conditions, two molecular species of the putative VIP binding unit were identified as broad autoradiographic bands of 80 000 and 56 000 daltons (Da). Both the large and small species showed the same high affinity for ^{125}I-VIP binding and subsequent cross-linking (half-maximal inhibition at 3 nM unlabeled VIP). The 80-kDa species was partially converted to the 56-kDa form by denaturing conditions and was extensively degraded when incubated at 20 °C for 30 min with 1 μg/mL chymotrypsin, trypsin, or elastase to fragments that migrated similarly to the 56-kDa unit. In contrast, the 56-kDa moiety was resistant to attack by serine proteases. Both the 80- and 56-kDa species were microheterogeneous due at least in part to the presence of carbohydrate chains, each species binding fractionally to wheat germ agglutinin (WGA)–agarose (~50%). The WGA-bound fraction (eluted with *N*-acetylglucosamine) was relatively retarded on acrylamide gels as compared to the WGA-unbound fraction. Exposure of the 80- and 56-kDa species to endo-β-acetylglucosaminidase F reduced the apparent molecular mass of each by 19 kDa, indicating the presence of complex N-linked carbohydrate chains. The receptor species do not appear to have high-mannose N-linked chains since they did not interact with concanavalin A and were not cleaved by endo-β-acetylglucosaminidase H. The ^{125}I-VIP-labeled receptor was readily solubilized with 1% Triton X-100 and eluted from Fractogel 55F as a 200-kDa unit and a 47-kDa nonglycosylated fragment that was an artifact of the solubilization process. Both the 80- and 56-kDa species were components of the solubilized receptor. The hepatic VIP receptor appears to be a large glycoprotein probably consisting of one or more 77-kDa units (80 kDa less the mass of the cross-linked VIP), 19 kDa of which is accounted for by complex N-linked carbohydrate chains. This VIP binding unit is complexed noncovalently either with itself or with one or more modifying subunits. A smaller 53-kDa moiety, though possibly a true companion to the larger binding subunit, is more likely to be a partially degraded form of the receptor. The hepatic VIP receptor is structurally distinct both from the hepatic glucagon receptor and from the VIP receptors in the intestinal enterocyte and in human lymphoblasts.

Vasoactive intestinal polypeptide (VIP),[1] a neurotransmitter and hormone, has widespread actions on many tissues. Commensurate with this ubiquitous role, VIP receptors have been identified in the gastrointestinal tract (salivary gland, gastric smooth muscle, gastric cells, intestinal epithelial cells, pancreatic acini and islets), the central nervous system, the lung, the heart, the blood vessels, the uterine smooth muscle, the adrenal gland, the retina, the adipocytes, and different types of lymphocytes (Said, 1984).

In the gastrointestinal system, VIP acts on smooth muscle to influence motility and induces secretion of intestinal and pancreatic fluid and electrolytes. The physiologic role of VIP in the liver is uncertain. While early evidence suggested a role of glucose production and glycogenolysis (Said & Mutt, 1970; Kerins & Said, 1973; Souquet et al., 1982), relatively high concentrations of the hormone are required to produce these metabolic effects (Wood & Blum, 1982; Bataille et al., 1974). Indeed, the major role of the liver may be clearance of the peptide from the portal circulation (Chayvialle et al., 1981; Gammeltoft et al., 1984). Experiments both with isolated hepatocytes and liver plasma membranes, however, have shown convincing kinetic evidence for a liver VIP receptor (Gammeltoft et al., 1984; Desbuquois et al., 1973; Desbuquois, 1974). More recently, by cross-linking ^{125}I-VIP to liver cell plasma membranes, Couvineau & Laburthe (1985) have identified 48 000- and 86 000-dalton (Da) macromolecules that interact very similarly with the hormone, the smaller of which they believe is the authentic binding component of the receptor. On the basis of the use of three different covalent cross-linking reagents, we now report that the solubilized receptor from rat liver membranes is a large macromolecule (~200 kDa) containing two molecular forms (80 and 56 kDa) of the cross-

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[1] Abbreviations: VIP, vasoactive intestinal peptide; BSA, bovine serum albumin; DSS, disuccinimido suberate; DSP, disuccinimido dithiobis(propionate); HSAB, succinimido 4-azidobenzoate; NP-40, Nonidet P-40; SDS, sodium dodecyl sulfate; WGA, wheat germ agglutinin; TCA, trichloroacetic acid; Endo F, endo-β-acetylglucosaminidase F; Endo H, endo-β-acetylglucosaminidase H; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; Con A, concanavalin A.

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linked receptor. Both forms are polydisperse in denaturing gels due to the presence of a 19-kDa N-linked carbohydrate component. The 80-kDa form, though very sensitive to protease cleavage, is the better candidate for the true binding subunit of the hepatic VIP receptor.

Experimental Procedures

Chemicals and Reagents. Synthetic VIP, secretin, glucagon, beef insulin, and aprotinin were obtained from Sigma. [3-Iodo[^{125}I]tyrosyl]VIP was from Amersham. The covalent cross-linkers DSS, DSP, and HSAB were from Pierce. Fractogel TSK 50F and TSK 55F were from EM Science. Concanavalin A–agarose and WGA–agarose were from Vector. Endo F (*Flavobacterium meningosepticum*) was from New England Nuclear, and Endo H (*Streptomyces plicatus*) was from Miles. Triton X-100 was from Sigma, NP-40 was from Particle Data, acrylamide and *N*,*N*′-methylenebis-(acrylamide) were from Bio-Rad, and SDS was from BDH Chemicals. Molecular weight standards were from Bio-Rad and Pharmacia.

Preparation of Liver Plasma Membranes and VIP Binding. Liver plasma membranes were obtained from male Sprague-Dawley rats according to steps 1–11 outlined by Neville (1968). This method yielded a 50–200-fold enrichment in the VIP binding capacity and an 18-fold enrichment in alkaline phosphatase, as compared to the original crude homogenate. Membrane protein was determined by Coomassie blue dye binding by use of reagent from Bio-Rad.

For binding studies, up to 200 μg of liver plasma membrane protein was incubated with radioactive VIP at a final concentration of 2.5×10^{-11} (for binding experiments) or 2.5×10^{-10} M (for cross-linking experiments) in 1 mL of 100 mM sodium–potassium phosphate buffer (NaH_2PO_4 and K_2HPO_4), pH 7.5, containing 1.4% BSA, 0.05% Triton X-100, and 0.002% sodium azide in a 12 × 75 mm polyproplene tube for 1 h at 22 °C. This buffer had been centrifuged at 38000*g* for 35 min and filtered through a 0.20-μm Nalgene filter prior to the experiment. At the end of the incubation, VIP associated with the liver plasma membrane was separated from unbound VIP by centrifugation at 38000*g* for 15 min. Binding of VIP to the tube was less than 1.5% of the initial amount added.

Chemical Cross-Linking. The methods for chemical cross-linking of VIP to the receptor were modified from those previously described (Sakamoto et al., 1983, 1984; Laburthe et al., 1984). The pellet obtained from VIP binding experiments was washed once in either cold 100 mM sodium–potassium phosphate buffer, pH 7.4 (for cross-linking with HSAB), or 60 mM HEPES, pH 7.4 (for cross-linking with DSS or DSP). After resuspension in 1 mL of the same buffer, 10 μL of 100 mM cross-linker that had been freshly dissolved in Me_2SO was added. For DSS and DSP, cross-linking was facilitated by incubation for 15 min at 4 °C on a rotary shaker, and the reaction was stopped by addition of 2 mL of 60 mM ammonium acetate and 60 mM HEPES, pH 7.4. For HSAB, cross-linking involved exposure to a 275-W sunlamp at a distance of 12 cm for 15 min. In all cases, the treated membranes were harvested by centrifugation at 38000*g* for 15 min and then washed once in 100 mM sodium–potassium phosphate buffer, pH 7.8.

Solubilization. The cross-linked membrane pellets were dispersed by passage through a 25-gauge hollow needle in 1 mL of buffer A (10 mM Tris-HCl, pH 8, 100 mM NaCl, 1 mM $MgCl_2$, and 1 mM $CaCl_2$) containing 1 mg/mL bacitracin, 0.1 unit/mL aprotinin, and 1% w/v Triton X-100. The dispersed membranes were incubated in this detergent buffer overnight at 4 °C on a rotating shaker. Solubilized material was separated from residual membranes by centrifugation at 100000*g* for 1 h.

Gel Permeation Chromatography. Fractogel TSK 50F or TSK 55F were packed and run in buffer A containing 1% w/v Triton X-100. Samples were applied to the column through flow adaptors and eluted at 50 mL/h. Calibration standards were dextran blue, ferritin, BSA, cytochrome *c*, β-fructosidase, ^{125}I-VIP, and NaCl. Protein standards were identified by Bio-Rad protein assay, β-fructosidase was assayed enzymatically, and NaCl was determined by conductivity measurement.

Lectin Affinity Chromatography. One milliliter of agarose-bound WGA or concanavalin A, equilibrated with buffer A containing 0.2% Triton X-100, was placed in a Bio-Rad Econo-Column and the sample applied and washed with 20 mL of the same buffer. Material bound was then displaced with buffer A containing 0.2% Triton and either 300 mM *N*-acetylglucosamine for WGA or 10 mM methyl α-glucoside, followed by 500 mM methyl α-mannopyranoside, for concanavalin A.

Endoglycosidase Digestion. For Endo F digestion, the cross-linked membrane pellets (160 μg of protein) were resuspended in 100 μL of 10 mM sodium–potassium phosphate buffer, pH 6.1, 5 mM EDTA, 1% Nonidet P-40, 0.1% SDS, and 1% mercaptoethanol containing 2.5 units of Endo F (Elder & Alexander, 1982). Digestion was carried out for 1–24 h at 37 °C. The mixture was then diluted to 1 mL with deionized water containing BSA and ovalbumin (100 μg each) to enhance protein recovery, and TCA was added to a final concentration of 8% w/v. The mixture was kept at 4 °C for 30 min and centrifuged for 5 min in a microfuge and the precipitate washed twice with cold acetone and once with sodium–potassium phosphate buffer, pH 7.8. A control sample was submitted to the same treatment, except that Endo F was omitted.

The technique for Endo H digestion was the same as that for Endo F except that 100 μg of membrane protein was incubated with 50 milliunits of the glycosidase for 16 h in 1 mL of 150 mM sodium citrate, pH 5.5 (Ahnen et al., 1983).

SDS–Acrylamide Electrophoresis and Autoradiography. Membrane pellets or TCA precipitates were resuspended in 200 μL of 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 1 mM EDTA, and 10 mM DTT (buffer B). DTT was omitted when DSP was used as the cross-linking agent. After incubation for 30 min at 37 °C, the material was applied to a 16 cm × 14 cm × 1.5 mm gel in a Bio-Rad multislab apparatus. The 12-cm separation gel was 10% acrylamide and 2% cross-linked, and the overlaying 1.5-cm stacking gel was 3.6% total acrylamide and 2% cross-linked. The buffer system of Laemmli (1970) was used, except that 0.05% SDS was included in the upper reservoir and no SDS was present in the gel or lower reservoir buffer. Electrophoresis was carried out initially with 60 V (stacking gel) and then with 120 V (separating gel). Gels were fixed in 25% isopropanol and 10% acetic acid, stained with Coomassie blue, immersed in 5% glycerol, and dried under vacuum. Kodak XOmat AR films were exposed at −70 °C in cassettes fitted with Du Pont Cronex Lightning Plus high-speed enhancing screens. In some experiments the protein bands were localized with the autoradiogram as template and then were excised from the dried gel for direct quantitation of radioactivity.

Electroelution of VIP–Receptor. After electrophoresis, the radioactive bands, localized by the radioactivity in slices cut from a 2.0-cm vertical gel strip, were excised from the slab gel, placed on a 0.5-cm cylindrical 10% acrylamide gel, and

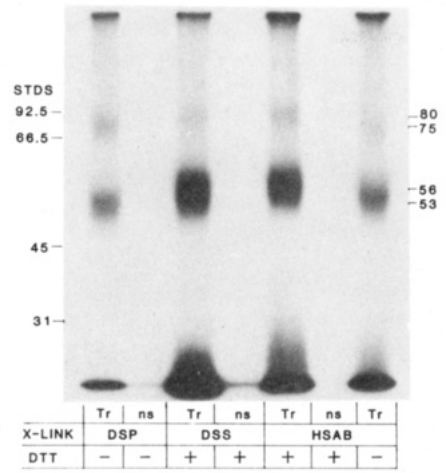

FIGURE 1: Acrylamide electrophoresis audioradiogram of liver plasma membranes treated with cross-linking reagents (DSP, DSS, or HSAB) after exposure to 2.5×10^{-10} M ^{125}I-VIP (trace, Tr) or ^{125}I-VIP plus 10^{-6} M unlabeled VIP (nonspecific, ns) as detailed under Experimental Procedures. Prior to electrophoresis, the labeled and cross-linked (×-link) membranes were suspended in 2% SDS with (+) or without (−) the addition of 10 mM DTT. Molecular weight standards were phosphorylase, bovine albumin, ovalbumin, and carbonic anhydrase. The apparent molecular weights of the two candidate receptor units, based on log–linear plots of R_f vs. mass, are shown on the right.

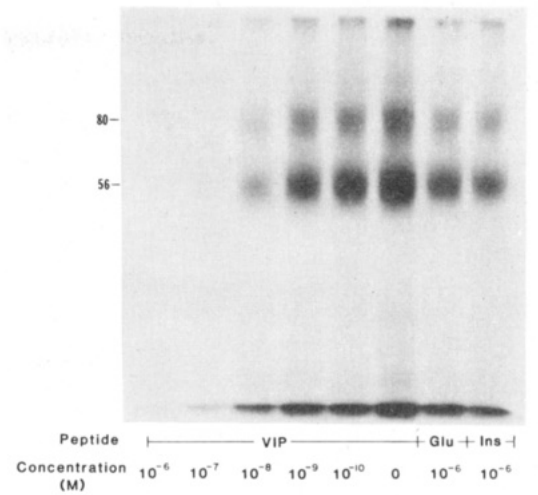

FIGURE 2: Specificity of the cross-linking of VIP to its receptor. Increasing concentrations of unlabeled VIP, insulin (Ins), or glucagon (Glu) were added to 60 μg of membrane protein/mL along with ^{125}I-VIP (2.5×10^{-10} M). After DSP cross-linkage and incubation in buffer B for 30 min at 37 °C, acrylamide electrophoresis–autoradiography was performed as detailed under Experimental Procedures.

electroeluted for 20 h at 2 mA into a spectrophor dialysis bag (Spectrum Medical Industries, exclusion M_r 12000–14000) that had been tied to the outlet of the glass cylinder containing the gel (Iyengar & Herberg, 1984; Cleveland et al., 1977). The recovered radioactive protein was concentrated in a microconcentrator (Centricon 10 from Amicon).

Results

Specific Binding of VIP to Rat Liver Plasma Membranes. Preliminary experiments with ^{125}I-VIP and liver plasma membranes revealed that maximal specific binding occurred at 22 °C after 40 min of incubation and was maintained until at least 75 min. Binding at 4 °C was slower (maximum at 90 min) but was maintained longer (150 min). Specific binding of VIP was a linear function of the amount of plasma membrane protein added from 1 to 150 μg. The maximal specific binding was 75% at 200 μg of membrane protein/mL of assay; nonspecific binding (after addition of 10^{-6} M unlabeled VIP) was 4.5% of the total binding and 3.3% of the specific binding. Red blood cell membranes showed no specific binding up to 100 μg of protein. When 2.5×10^{-11} M ^{125}I-VIP was exposed to liver plasma membranes in the presence of varying concentrations of unlabeled VIP, half-maximal inhibition occurred at 0.5 nM; a 250 times higher concentration was required for secretin to yield a similar half-maximal inhibition of binding. At concentrations 4 orders of magnitude higher than that required for VIP for half-maximal inhibition, glucagon and insulin competed only marginally for ^{125}I-VIP binding. When analyzed by the transformation of Scatchard (1949), these data were consistent with a single class of binding sites with an affinity constant of 1.0 ± 0.25 nM ($n = 3$) and a capacity of 5.0 ± 0.9 pmol/mg of protein ($n = 3$). An alternative two binding site model did not fit the experimental data as well as the single-site model (data not shown).

Cross-Linking of ^{125}I-VIP to Its Receptor and Analysis by Acrylamide Electrophoresis–Autoradiography. As assessed by SDS–polyacrylamide electrophoresis followed by autoradiography, all three cross-linking reagents, DSP, DSS and HSAB, promoted covalent cross-linking of VIP to macromolecules on liver plasma membranes. Analysis of the plasma membranes after cross-linking in the presence of ^{125}I-VIP revealed a complex autoradiographic pattern (Figure 1). Two radioactive bands appeared to be candidates for the true VIP receptor: in the absence of DTT, both the DSP and HSAB cross-linkers yielded species that migrated at M_r 75000 and 53000, while in the presence of DTT, the DSS and HSAB cross-linkers produced species migrating at M_r 80000 and 56000. Although this raises the possibility of intrachain disulfide bonds, the apparent retardation in the presence of reducing agents is not consistent with the existence of intersubunit disulfide bonds. All three cross-linking agents gave similar results. Radioactivity at the gel front could be accounted for by free iodine and un-cross-linked VIP. Radioactivity at the top of the separation gel probably represents aggregated material due to denaturation because it did not enter a 6% acrylamide separation gel (data not shown) and increased at higher temperature and with longer incubation with 2% SDS prior to electrophoresis (not shown). The rapidly moving bands that were included in the separation gel (cf. Figures 1, 2, and 7; 15000–30000-kDa bands just above gel front) appear to be fragments of the 80- and 56-kDa peptides because they were present in greater abundance with the most stringent denaturing conditions (100 °C for 20 min, not shown).

The specificity for binding and cross-linking was assessed by addition of increasing amounts of unlabeled VIP to the ^{125}I-VIP trace (2.5×10^{-10} M), followed by treatment with the cross-linking agent. Analysis by SDS–acrylamide electrophoresis and autoradiography showed competition for ^{125}I-VIP binding (Figure 2). Quantitation of radioactivity in the excised bands revealed that half-maximal inhibition of ^{125}I-VIP binding and cross-linking occurred at approximately 3 nM unlabeled hormone for both the 80- and 56-kDa species. High concentrations of glucagon and insulin produced minimal inhibition of ^{125}I-VIP binding and cross-linking to its receptor (Figure 2, right).

Interrelationship of the Major Cross-Linked Macromolecules. Because the 56-kDa receptor species may be a breakdown product of the larger 80-kDa species, we designed studies to examine the relationship of the two species. Each cross-linked species was excised from SDS gels and electroeluted through a 10% cylindrical acrylamide gel into an attached dialysis bag, as described under Experimental Procedures. The

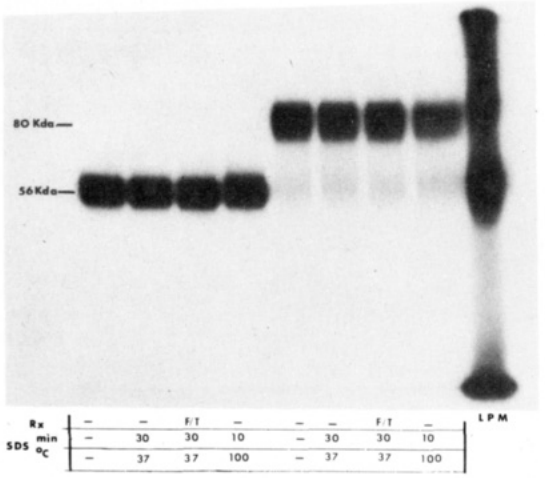

FIGURE 3: Recovery and reelectrophoresis of the 80- and 56-kDa species of ^{125}I-VIP–receptor units. After DSP cross-linkage of ^{125}I-VIP to liver membranes, the 80- and 56-kDa bands were excised, electroeluted, concentrated, and reapplied to a second SDS slab gel as detailed under Experimental Procedures. Prior to electrophoresis, each species was subjected to denaturation in buffer B for the period and temperature shown at the bottom of the figure. Some samples were also rapidly frozen and thawed (F/T) in liquid nitrogen prior to denaturation. LPM denotes the overexposed liver membrane starting material from which the species were isolated on the first electrophoresis. Notably, there was partial breakdown of the 80-kDa species to a product migrating at the position of the 56-kDa species under all conditions of denaturation with some augmentation with longer periods of time at higher temperatures.

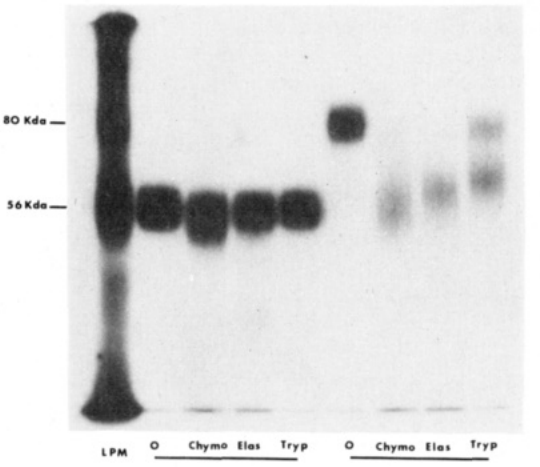

FIGURE 4: Cleavage of the 80- and 56-kDa VIP–receptor species by proteases. Each species, isolated from an acrylamide slab gel as described in the Figure 3 legend and under Experimental Procedures, was exposed to a single serine protease (1 μg/mL) for 30 min at 22 °C and examined by acrylamide electrophoresis–autoradiography. LPM denotes liver membrane control starting material. The left-hand lanes show the 56-kDa species and the right-hand lanes the 80-kDa species after exposure to protease-free buffer (O), chymotrypsin (Chymo), elastase (Elas), or trypsin (Tryp). Note the much greater sensitivity of the 80-kDa species to protease attack.

isolated 80- and 56-kDa species were then concentrated, subjected to different degrees of denaturation (Figure 3), and then reexamined by SDS electrophoresis–autoradiography. This electrophoretic isolation of the 80-kDa moiety was associated with its partial spontaneous conversion to a band that comigrated with the 56-kDa moiety. This conversion was augmented somewhat with more stringent denaturing conditions (e.g., exposure to 2% SDS for 30 min at 37 °C or for 10 min at 100 °C) (Figure 3).

The effect of proteases on the 80- and 56-kDa moieties is shown in Figure 4. The 80-kDa moiety was exquisitely sensitive to the serine proteases, as little as 1 μg/mL at 22 °C for 30 min cleaving it to smaller fragments. Notably, chymotrypsin yielded a product that migrated similarly to the 56-kDa species. On the other hand, the 56-kDa species was resistant to protease attack, only chymotrypsin being able to marginally increase its migration under the same conditions.

In the aggregate, these experiments suggest that the native VIP receptor is a macromolecule of approximately 77 kDa (80-kDa VIP–receptor complex less 3 kDa for VIP) that is highly sensitive to protease. The 56-kDa species is most likely a proteolytic cleavage product of the larger receptor unit.

Microheterogeneity of the VIP Receptor. Both species of the VIP–receptor complex migrated in SDS electrophoretic systems as relatively broad bands. Because this pattern might be produced by polydispersity of the receptor units, the upper and lower halves of each band from SDS gels were excised, electroeluted, and reexamined by SDS electrophoresis–autoradiography. As shown in Figure 5, the upper and lower portions of both the 80- and 56-kDa species were readily separable, indicating that two or more subspecies accounted for the relatively broad bands found on SDS gels. This microheterogeneity is probably related to variable degrees of glycosylation of the receptor (cf. section on lectin binding and endoglycosidase action, below).

Solubilized VIP Receptor from Liver Plasma Membranes. After ^{125}I-VIP was bound to plasma membranes and cross-

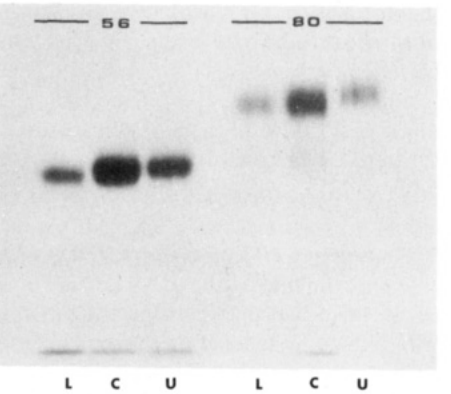

FIGURE 5: Heterogeneity of the VIP–receptor complexes. After DSP cross-linking and SDS electrophoresis, the broad 56- and 80-kDa radioactive bands were excised into upper and lower halves, electroeluted, and examined by SDS electrophoresis as detailed in previous figure legends and under Experimental Procedures. C denotes entire 56- or 80-kDa band; L, lower portion of band; U, upper portion of band. In addition to the obvious heterogeneity of both the small and large species, the 80-kDa moiety showed partial spontaneous breakdown to the 56-kDa fragment.

linked with HSAB, the mixture was treated with 1% Triton X-100 in buffer A for 16 h at 4 °C. An appreciable proportion (40–60%) of the bound radioactivity was recovered in the supernatant after centrifugation for 100000g for 1 h.

Examination of the solubilized material by gel filtration on a Fractogel 55F column revealed several peaks of radioactivity (Figure 6). The void volume peak appeared to constitute aggregated material since it increased substantially when Triton was omitted from the eluent buffer (data not shown). This was not examined further. The two major peaks that were retarded on the column (200 and 47 kDa) were subjected to SDS electrophoresis as shown in Figure 7. The 200-kDa peak yielded the typical large (M_r 80000) and small (M_r 56000) species found when VIP-cross-linked membranes were applied directly to SDS gels. The 47-kDa peak migrated as a single species (~45 kDa on SDS electrophoresis). That this smaller species is an artifact of the solubilization process can be seen from analysis of the Triton-treated membranes prior

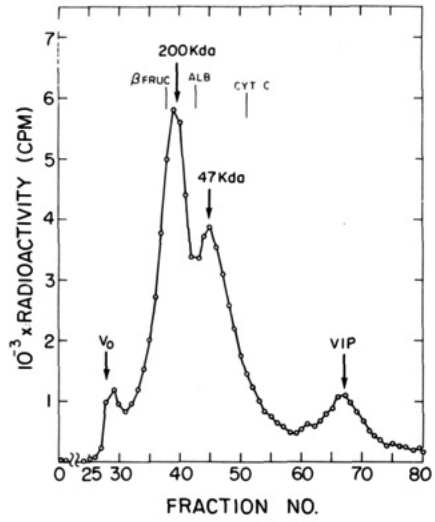

FIGURE 6: Gel filtration chromatography of the solubilized VIP–receptor complex. Liver plasma membranes (540 μg), cross-linked with HSAB, were solubilized with 1% Triton X-100 and applied to a 45 × 1.5 cm column of Fractogel 55F as detailed under Experimental Procedures. The column was eluted with buffer A containing 1% Triton X-100 and collected in 1-mL fractions. V_0 is the void volume. The standard proteins were as follows: β-fructosidase (βFRUC), M_r 270 000; bovine albumin (ALB), M_r 66 200; and cytochrome (CYT C), M_r 12 400. VIP denotes the eluted position of VIP (M_r 3300). The 200- and 47-kDa species of radioactive VIP–receptor complex were recovered and examined further by SDS electrophoresis (cf. Figure 7).

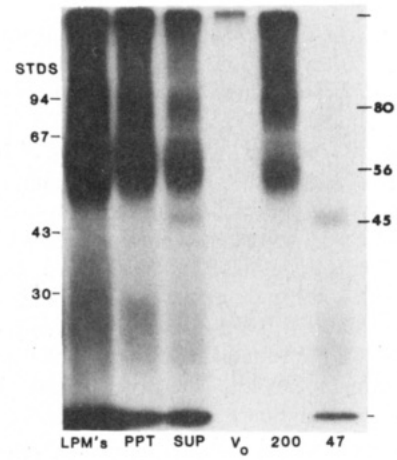

FIGURE 7: Acrylamide electrophoresis–autoradiography of solubilized VIP–receptor complex. After HSAB cross-linkage with ^{125}I-VIP, the liver membranes (LPM's) were solubilized (SUP lane) as described (cf. Figure 6 legend and Experimental Procedures). PPT denotes residual precipitate after solubilization; V_0, void volume; 200, the 200-kDa peak; 47, the 47-kDa peak isolated from gel filtration experiment (Figure 6).

to gel filtration (Figure 7, SUP lane). Notably, most of the radioactivity associated with the solubilized 47-kDa species was not covalently bound and migrated in the gel front after SDS treatment.

Binding of ^{125}I-VIP–Receptor Complex to WGA. After being cross-linked with DSP and solubilized with either 0.5% NP-40 or 1% Triton X-100, the sample was applied to a 1-mL column of WGA–agarose. When 20 mL of buffer A containing 1% Triton X-100 was passed through the column, followed by 20 mL of the same buffer containing 300 mM *N*-acetylglucosamine, approximately half of the radioactive VIP–receptor complex passed unbound, and the remainder was bound to the immobilized WGA and readily eluted by the addition of *N*-acetylglucosamine. Examination of these

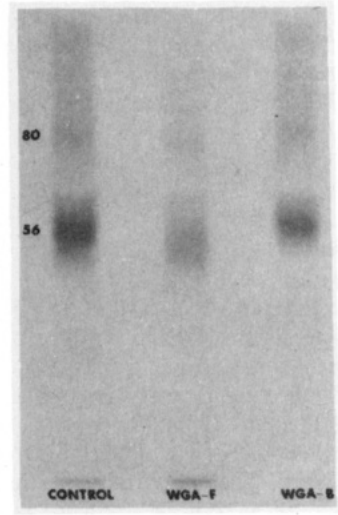

FIGURE 8: Comparison of VIP–receptor species that were bound and eluted from WGA–agarose (WGA-B) with those not retarded by the immobilized lectin (WGA-F). The 200-kDa ^{125}I-VIP species isolated from gel filtration chromatography (cf. Figure 7) served as the starting material (control). Albumin and ovalbumin, 100 μg each, were added to pooled eluent to ensure complete recovery, and the mixture was made 8% in TCA; the precipitate was applied to the SDS–acrylamide gel, as detailed under Experimental Procedures.

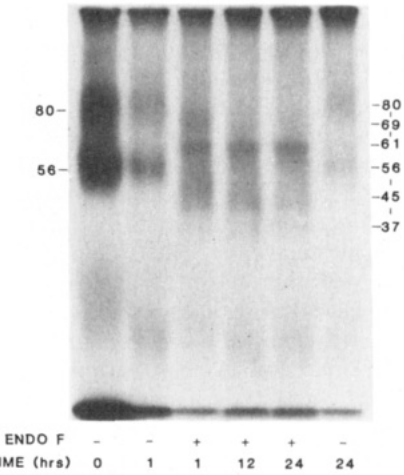

FIGURE 9: Endo F treatment of the ^{125}I-VIP–receptor complex in liver plasma membranes. Liver plasma membranes (160 μg of protein), cross-linked with HSAB, were exposed to 2.5 units of Endo F for 1–24 h and applied to SDS gels as described under Experimental Procedures. The designation at the base of the autoradiograph denotes the presence (+) or absence (−) of Endo F for the incubation periods shown. The 80-kDa species is converted to a 61-kDa product and the 56-kDa species to a 37-kDa species over the 24-h period of incubation.

fractions by SDS–acrylamide electrophoresis revealed that the 80- and 56-kDa bands were confined to the bound fraction (data not shown). When the individual 200- and 47-kDa peaks from the Fractogel 55F column (cf. Figure 6) were applied to WGA, the 47-kDa species passed unbound, but about 50% of the 200-kDa species bound to the lectin column and was eluted with *N*-acetylglucosamine (data not shown). When the unbound and bound fractions of the 200-kDa species were TCA-precipitated along with 100 μg each of BSA and ovalbumin as carrier proteins and studied on SDS–acrylamide gel electrophoresis, there was relative retardation of the 56- and 80-kDa species of the WGA-bound fraction as compared to the unbound fraction (Figure 8). This suggests that the microheterogeneity of the VIP receptor is accounted for to a great extent by variation in the composition of its carbohydrate chains. The solubilized material from ^{125}I-VIP-cross-linked

membrane did not bind to a 1-mL column of concanavalin A–agarose (data not shown).

Treatment of the Cross-Linked Receptor with Endoglycosidases. The fact that the solubilized receptor bound to WGA and was eluted from WGA by *N*-acetylglucosamine (GlcNAc) suggested that it may contain carbohydrate chains with GlcNAc residues. This was further analyzed by digestion with endoglycosidases. After cross-linking, the liver membranes were treated with up to 50 units of Endo H for 18 h at 37 °C. The SDS electrophoretic pattern was unchanged after exposure to Endo H, confirming the Con A binding experiments suggesting that high-mannose carbohydrate chains are not constituents of the receptor (data not shown). When 160 μg of cross-linked membranes were treated with 2.5 units of Endo F for 1, 12, and 24 h, migration of the 80- and 56-kDa species was altered. Analysis of Figure 9 reveals that the 80-kDa species was sequentially reduced in size to 69 kDa and then to 61 kDa. The 56-kDa species was converted initially to 45 kDa and eventually to 37 kDa. Thus, both the 80- and 56-kDa species appear to contain more than one N-linked carbohydrate chain, and each species has a carbohydrate mass of approximately 19 kDa. These findings were verified by examination of the effect of Endo F on the individual 80- and 56-kDa species of the receptor (not shown).

DISCUSSION

Molecular Size of the Cross-Linked Receptor Units. As has been shown in previous studies of rat liver membranes (Bataille et al., 1974; Desbuquois et al., 1973; Desbuquois, 1974), there is a high-affinity binding site for VIP in purified liver plasma membranes that can be competitively blocked by nanomolar concentrations of nonlabeled hormone. The high affinity of the hormone for its receptor is analogous to that found for VIP receptors on other gastrointestinal membranes including the intestinal enterocyte (Prieto et al., 1979; Amiranoff et al., 1980) and pancreatic acinar cells (Jensen & Gardner, 1981). By use of three different cross-linking reagents, we have identified two molecular forms of the liver membrane VIP–receptor complex with M_r's of 80 000 and 56 000 (Figures 1 and 2). Although the molecular characteristics of the VIP receptor on pancreatic cells are not yet known, cross-linking studies of intestinal membranes have shown two types of VIP-binding macromolecules (high affinity, 73 kDa, and low affinity, 33 kDa) that are distinctly different from those found in liver membranes (Couvineau & Laburthe, 1985; Laburthe et al., 1984). The only other cross-linked VIP–receptor studied thus far, from human lymphoblasts (Wood & O'Dorisio, 1985), displays important differences from the hepatic receptor; the lymphoblast receptor has appreciable affinity for glucagon, is not readily cross-linked by HSAB, and has a smaller mass (~50 kDa) than the corresponding hepatic species.

When the mass of the cross-linked hormone is deducted, the putative binding subunits of the hepatic VIP receptor have molecular masses of 77 000 and 53 000 Da. These moieties are similar to those recently identified by Couvineau & Laburthe (1985), but the larger species was 10 kDa smaller than they had reported. However, by reconstructing log–linear plots from gel electrophoretic data in Figure 1 of Couvineau & Laburthe (1985), we obtained molecular sizes for the VIP–receptor complex of 82 and 55 kDa, values not significantly different from those that we now report (80 and 56 kDa). Although neither the large nor the small VIP-binding macromolecule appears to contain interchain disulfide bonds, their more rapid migration on SDS gels under nonreducing conditions (cf. Figure 1) suggests that intrachain disulfide bonding may maintain a conformation that retards migration in SDS gels. Indeed, disulfide bonding may be important for VIP binding, since exposure of liver membranes to DTT prevents subsequent binding of VIP (data not shown).

Polydispersity of the Receptor Units: Relationship to Carbohydrate Chains. The relatively broad banding pattern found in SDS gels prompted us to recover the upper and lower portions of the VIP receptor bands and to reanalyze them on SDS gels. Figure 5 clearly shows that the upper and lower regions of both the 80- and 56-kDa species migrate as individual bands, verifying that there are at least two, and probably several, molecular forms of each receptor unit after cross-linking VIP to liver plasma membranes. This microheterogeneity appears to be related to the variation of carbohydrate chain composition of the receptor, on the basis of the correlation of larger molecular forms with binding affinity for WGA–agarose and of low molecular species with those that do not interact with the immobilized lectin (Figure 8). Finally, the fact that Endo F removal of the N-linked chains yielded a more narrowly migrating band on SDS gels in consistent with the concept that variation of the number or mass of carbohydrate chains is responsible for heterogeneity of both the 80- and 56-kDa species (cf. Figure 9). The VIP–receptor complexes previously identified in intestinal membranes, though of different molecular size than those in liver, also migrated as very broad bands on SDS gels (Laburthe et al., 1984). By analogy, the intestinal receptors may also contain carbohydrate chains that confer polydispersity, but this possibility has not yet been examined.

80- and 56-kDa Candidates for the Authentic Receptor Subunit. In the only previous study of cross-linking VIP to liver plasma membranes, Couvineau & Laburthe (1985) noted that the cross-linked VIP–receptor complex in liver plasma membranes displayed maximal radioactivity in the smaller 56-kDa unit. Because of this, they proposed that, rather than being a proteolytic product of the larger cross-linked moiety, it was more likely to be the true binding subunit. Yet they noted a greater affinity of GTP for the larger moiety in terms of the nucleotide's ability to displace VIP from the receptor. On the basis of data with ^{125}I-VIP and three different cross-linking agents, we have found that both the 80- and 56-kDa species become highly labeled. The larger species is exquisitely susceptible to proteolytic cleavage to smaller fragments including those that migrate similarly to the 56-kDa species (cf. Figure 4). Furthermore, when the 80-kDa moiety is recovered, concentrated, and reapplied to SDS gels, it is partially cleaved to a product migrating identically with the smaller 56-kDa form (Figure 3). Since both forms have the same affinity for VIP and contain the same mass of carbohydrate, it seems likely that the 56-kDa form is derived by removal of a 24-kDa peptide fragment that contains neither the VIP binding site nor carbohydrate chains. Alternatively, the 80-kDa species may represent the 56-kDa VIP binding unit that has been covalently linked to a 24-kDa modifying subunit during the VIP cross-linking step. This would require a very close spatial association of such a modifying subunit to allow cross-linking, a feature that has apparently not been observed for other hormone receptors. Of course, the possibility that both the large and small receptor units are native to liver membranes remains, but it seems more likely that the 56-kDa moiety is an artifact produced by cleavage of the larger form during the process of membrane preparation or chemical cross-linking.

Solubilized VIP–Receptor Complex. There are no previous studies characterizing the liver membrane VIP receptor after solubilization. We have estimated the molecular size of the

solubilized hepatic VIP receptor by gel filtration, and the apparent molecular mass of 200 000 Da may include detergent molecules that are complexed with membrane glycoproteins to varying degrees. However, the receptor was readily solubilized by nonionic detergents and, hence, may not be highly hydrophobic. Also of note is the fact that the 47-kDa VIP receptor fragment generated by the solubilization process appears to be identical in molecular size by both denaturing gel electrophoresis and nondenaturing gel filtration of the Triton-solubilized material (cf. Figures 6 and 7). Thus, the approximate mass of 200 kDa for the VIP–receptor unit is probably a reasonable estimate of the molecular size of the native receptor.

The solubilized liver VIP–receptor unit is distinct from those found for other hormones of similar structure or biologic function. Thus, the liver membrane glucagon receptor appears to be a homodimer of approximately 120 kDa consisting of two binding subunits (Iyengar & Herberg, 1984; Herberg et al., 1984); the cholecystokinin receptor is a disulfide-bonded heterodimer of 120 kDa (Sakamoto et al., 1983, 1984). Although composed of a distinctly smaller binding subunit, the glucagon receptor does contain an 18-kDa N-linked carbohydrate moiety that is of similar size to that we have shown for the binding subunit of the VIP receptor (Iyengar & Herbert, 1984). Of all the hormone receptors that have been characterized thus far, only unrelated receptors, such as those for insulin and epidermal growth factor (EGF), are of similar size.

The finding of a 47-kDa moiety, in addition to the 80- and 56-kDa receptor units after solubilization, suggests that detergent treatment of the membranes must release yet a smaller fragment which dissociates from the major solubilized VIP receptor in nondenaturing conditions (Figure 6) and does not interact with WGA. Despite the fact that VIP apparently remains cross-linked to this smaller fragment during solubilization and gel filtration, the majority of the radioactivity is subsequently released from the 47-kDa fragment when it is subjected to SDS electrophoresis (Figure 7). Whether this instability is related to cleavage of the 47-kDa peptide at a position near the VIP binding site or to breakdown of the ^{125}I-VIP itself cannot be determined from our studies. In other experiments not detailed here, we have found that the 47-kDa receptor unit probably does not retain any N-linked carbohydrate chains. It is tempting to speculate that the 47-kDa fragment is produced when one or more additional cleavage steps occur by virtue of protease attack on an intramembrane site that becomes available only after removal of the VIP binding subunit from the membrane. This finding may eventually facilitate studies of the localization and topography of the VIP subunit within the lipid bilayer.

Because the liver consists of bile ductular and reticuloendothelial (Kupffer) cells as well as parenchymal cells, it is uncertain which cell type is the predominant location of the hepatic VIP receptor. Recently, Gardner et al. (1985) have observed appreciably more binding of VIP to Kupffer cells than to hepatic parenchymal cells in a collagenase-dispersed preparation. In studies not detailed here, however, we have found a 17-fold decrease in VIP binding to liver plasma membranes after exposure to collagenase (specific binding: buffer control 50%, buffered collagenase 3%). Hence, the vast majority of hepatic plasma membrane receptors appear to be rendered nonfunctional by conditions necessary to disperse and isolate cell types. Because the plasma membrane fraction derived directly from the liver without collagenase treatment contains a specific parenchymal cell antigen (Neville, 1968), we believe that the receptor most likely resides on hepatocytes. A final answer on the exact location of the hepatic VIP membrane receptor must await localization by specific anti-receptor antibodies.

In essence, in its native state the VIP receptor appears to be a large glycoprotein complex that probably is made up of one or more 77-kDa binding subunits (80 kDa less the mass of the cross-linked VIP) and that includes a 19-kDa unit of N-linked complex carbohydate chains. The binding subunit appears to be associated in oligomeric form, either with itself or with one or more modifying subunits. This receptor is structurally distinguishable from VIP receptors in other locations in the gastrointestinal tract and may have a special functional role in the liver, such as clearance of the circulating hormone.

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Adenosine 5′-Triphosphate Synthesis in *Escherichia coli* Submitted to a Microsecond Electric Pulse†

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ABSTRACT: The total cytoplasmic ATP content (bound and free) increased in *Escherichia coli* when the bacteria were submitted to electric pulses with field strengths of 1–6 kV/cm and a decay time of 7–20 μs. The electron-transport chain was blocked by cyanide, and ATP synthesis was detected by a luminescence assay. The amount of newly formed ATP depends on the field strength. A total of 150 pmol of ATP was formed per milligram of bacteria submitted to a 3 kV/cm pulse. Synthesis was blocked by uncouplers and ionophores (valinomycin). The F_1F_0-ATP synthase inhibitor dicyclohexylcarbodiimide blocked a large part of this synthesis. Synthesis was not induced in unc mutants (unc B, unc D). The synthesis of ATP is related to the induced transmembrane potential, not to the Joule heating. A minimum 35–50-mV increase in membrane potential must be maintained for at least 12 μs to trigger this synthesis. This very fast energy transduction in bacteria is in good agreement with our previous results concerning submitochondrial particles. Because of the localized character of the induced membrane potential, these results are in agreement with the recent hypothesis of "mosaic proton coupling".

Conversion of the energy formed during respiration into the terminal phosphate bond of ATP[1] has been the focus of numerous studies using chloroplasts, mitochondria, or bacteria as experimental systems (Boyer et al., 1977). A striking similarity exists among all these systems not only in the enzymes involved (F_1F_0-ATP synthase) but in the mechanism of such a conversion (Boyer et al., 1977). The basic mechanism of energy transduction is postulated to be the conversion of the electrochemical gradient evoked by the respiratory chain into a chemical bond.

This hypothesis has been tested by the artificial creation of such a potential. If bacteria were first loaded with K^+ ions and then resuspended in a K^+-free medium, ATP synthesis was triggered in *Escherichia coli* and *Streptococcus lactis* by the addition of a small aliquot of the K^+ ionophore valinomycin (Maloney et al., 1974; Grinius et al., 1975). A complete analysis of this process in *E. coli* (Wilson et al., 1975) showed that a membrane potential was enough to induce the synthesis (no pH gradient was required). Furthermore, this last study (Wilson et al., 1975) demonstrated that the F_1F_0-ATP synthetase was involved in this conversion. Mutants where this enzyme was either lacking or inactive (unc) were unable to generate ATP when submitted to such a concentration pulse.

In these experiments, the ionic content of the cytoplasm was completely different from physiological conditions, and the consequences of this drastic change were unknown. Furthermore, the membrane structure was clearly affected by the ionophore. Recently, a direct electric alteration of membrane potential under less perturbing conditions has been developed (Kinosita & Tsong, 1977a; Teissié & Tsong, 1981a; Teissié et al., 1981). When a cell suspension is submitted to an electric field, a transmembrane potential is evoked. This potential is directly related to the external stimulus. Such a technique has been used in the study of ATP synthesis in chloroplasts (Witt et al., 1976), reconstituted bacterial systems (Rogner et al., 1979), and submitochondrial particles (Teissié et al., 1981; Knox & Tsong, 1984). Ionic transport against a concentration gradient can also be driven by such a stimulation (Teissié & Tsong, 1981b).

This paper shows that an external electric field may induce ATP synthesis in *E. coli*. This effect is associated with the induced membrane potential and not with a temperature increase. The study used electric pulses of various durations in the microsecond range. It provides evidence that energy transduction in the bacterial membrane is a very rapid process (several microseconds), which is triggered as soon as the membrane potential passes a critical threshold. Furthermore, this transduction is associated with a localized effect of the membrane potential in agreement with the "mosaic proton coupling" hypothesis (Westerhoff et al., 1984).

Materials and Methods

E. coli strains CB 0129 and AN 120 (unc A) were provided by Prof. Louarn (this institute). Mutants AN 719 (unc B)

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[1] Abbreviations: ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, *N,N′*-dicyclohexylcarbodiimide; DNP, 2,4-dinitrophenol; EDTA, ethylenediaminetetraacetic acid; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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