The rat liver vasoactive intestinal peptide binding site

Molecular characterization by covalent cross-linking and evidence for differences from the intestinal receptor

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To identify the molecular components of the vasoactive intestinal peptide (VIP) binding sites in the liver, ^{125}I -labelled VIP was covalently linked to liver membranes by using the cleavable cross-linker dithiobis(succinimidylpropionate). Purified rat liver plasma membranes were incubated with ^{125}I -VIP, washed and treated with 1 mM-cross-linker. Polyacrylamide-gel electrophoresis of membrane proteins followed by autoradiography revealed a major ^{125}I -VIP-protein complex of M_r 51 000. A minor M_r 89 000 complex was also observed. An identical pattern of protein labelling was obtained using crude membranes from rat liver. Labelling of the M_r 51 000 and 89 000 species was specific in that it could be abolished by native VIP, but was unaffected by $1\text{ }\mu\text{M}$ -glucagon and cholecystokinin octapeptide. Densitometric scanning of autoradiographs indicated that the labelling of the two species was abolished by similar low VIP concentrations (0.1–100 nM). It was also reduced by two VIP agonists, peptide histidine isoleucine amide and secretin, with a potency that is 1/7 and 1/200 that of native VIP, respectively. The guanine nucleotide GTP in the concentration range between 10^{-7} and 10^{-3} M reduces the labelling of the major M_r 51 000 protein and that of the minor M_r 89 000 protein, but with a slightly higher potency. Assuming one molecule of ^{125}I -VIP was bound per molecule of protein, a major M_r 48 000 protein and a minor M_r 86 000 protein were identified as components of the high-affinity VIP binding sites in liver. This contrasts markedly with the pattern of labelling of rat intestinal epithelial membranes, where a M_r 73 000 protein was identified as a high-affinity VIP receptor and a M_r 33 000 protein as a low-affinity VIP binding site [Laburthe, Bréant & Rouyer-Fessard (1984) *Eur. J. Biochem.* **139**, 181–187], suggesting structural differences between VIP binding sites in rat liver and intestinal epithelium.

It is now clearly established that VIP triggers biological responses through specific binding to receptors located at the surface of target cells (Laburthe *et al.*, 1978a). VIP receptors have been studied functionally in various tissues and often mediate an efficient stimulation of adenylate cyclase by the peptide (Amiranoff & Rosselin, 1982). This is the case for example in intestinal

epithelium, where the VIP receptors characterized in detail in plasma membranes (Amiranoff *et al.*, 1980a) and isolated cells (Prieto *et al.*, 1979) promote a dramatic activation of the cyclic AMP production system (Amiranoff *et al.*, 1978; Laburthe *et al.*, 1978a,b, 1979). In sharp contrast, the great number of high-affinity binding sites identified in liver (Desbuquois *et al.*, 1973; Bataille *et al.*, 1974; Desbuquois, 1974; Laburthe *et al.*, 1978a; Misbin *et al.*, 1982; Gammeltoft *et al.*, 1984) seems to be poorly involved in adenylate cyclase activation. Indeed, VIP induces a very low (Desbuquois *et al.*, 1973; Bataille *et al.*, 1974; Waelbroeck *et al.*, 1981; Guerrero *et al.*, 1981) or even negligible

Abbreviations used: VIP, vasoactive intestinal peptide; DTSP, dithiobis(succinimidylpropionate); SDS, sodium dodecyl sulphate.

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(Amiranoff *et al.*, 1980b) stimulation of adenylate cyclase activity in liver membranes and is almost ineffective at raising cyclic AMP levels in isolated liver cells (Laburthe *et al.*, 1978a; Feliu *et al.*, 1983). This corroborates various observations suggesting that VIP does not participate in the physiological control of hepatic glucose metabolism (Kerins & Said, 1973; Wood & Blum, 1982; Souquet *et al.*, 1982; Gammeltoft *et al.*, 1984). These observations are consistent with the idea that liver VIP binding sites may be involved, at least in part, in non-cyclic-AMP-dependent processes (Amiranoff *et al.*, 1980b), such as VIP extraction from the portal blood (Misbin *et al.*, 1982; Gammeltoft *et al.*, 1984) and may be different from the VIP receptors efficiently coupled to adenylate cyclase.

The present paper has two objectives. Firstly, by using the cross-linking procedure previously developed to characterize rat intestinal VIP receptors (Laburthe *et al.*, 1984), we have analysed the rat liver membrane components responsible for VIP binding, showing the involvement of a major M_r 48000 protein and a minor M_r 86000 one. Secondly, we have compared, within the same experiment, the pattern of membrane protein labelling by ^{125}I -VIP in liver and intestinal epithelium, the archetype of a target where VIP mobilizes the cyclic AMP production system (Laburthe & Dupont, 1982). Very different patterns are observed, suggesting some structural differences between VIP binding sites in the two tissues.

Experimental

Materials

Pig VIP was provided by Professor V. Mutt (Karolinska Institute, Stockholm, Sweden). ^{125}I -labelled VIP was prepared by the chloramine-T method (Hunter & Greenwood, 1962) at a specific radioactivity of 250 Ci/g as described (Laburthe *et al.*, 1977). It displays the same activity as native VIP in stimulating cyclic AMP accumulation in a cultured cell line (HT-29) which is highly and specifically sensitive to VIP (Laburthe *et al.*, 1978b). Synthetic pig secretin was a gift from Professors E. Wünsch and L. Moroder (Max Planck Institute, Martinsried, Germany). Peptide histidine isoleucine amide (Tatemoto & Mutt, 1980) was kindly provided by Dr. K. Tatemoto (Karolinska Institute, Stockholm, Sweden). Other compounds were obtained from the following sources: pig pancreatic glucagon, Novo (Bagvaerdt, Denmark); cholecystokinin octapeptide, Peninsula Laboratories (Belmont, CA, U.S.A.); DTSP, Pierce Chemical Co. (Rockford, IL, U.S.A.). The calibrating proteins were from

Bethesda Research Laboratories (Guthersburg, MD, U.S.A.). Other chemicals were of the highest purity commercially available.

Membrane preparations

Male Wistar rats (2 months old) fed *ad libitum* were used in all experiments. Purified liver plasma membranes were prepared following exactly the method described by Neville (1968) and represent step 11 of the method. They were used in most experiments. Crude liver membranes were also prepared by the following method. Liver was homogenized using an electric blender (Waring) for 1 min at high speed. The homogenizing buffer was 0.01 M-triethylamine, pH 7.5, containing 0.25 M-sucrose and 5 mM-EDTA. The homogenate was centrifuged for 10 min at 2600g and the resulting supernatant for 20 min at 20000g. The final pellet was washed in 60 mM-Hepes buffer, pH 7.5, and kept at -80°C until used. The crude membranes were used in the experiment shown in Fig. 5. Plasma membranes from isolated small intestinal epithelial cells were prepared as described in detail elsewhere (Amiranoff *et al.*, 1978). Membrane proteins were determined by the method of Bradford (1976) with bovine serum albumin as standard.

Binding of ^{125}I -VIP to membranes

Membranes (about 100 μg of protein/ml) were incubated as described (Amiranoff *et al.*, 1980a) for 30 min at 30°C with ^{125}I -VIP (0.3 nM) and when necessary other compounds in 2.5 ml of 60 mM-Hepes buffer, pH 7.5, containing 2% (w/v) bovine serum albumin and 0.1% (w/v) bacitracin. The reaction was stopped by adding 35 ml of ice-cold 60 mM-Hepes buffer, pH 7.5, and membrane-bound ^{125}I -VIP was obtained by centrifugation for 15 min at 20000g. The supernatant was withdrawn and the pellet used immediately for subsequent experiments.

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

Membranes containing bound ^{125}I -VIP were suspended in 1 ml of 60 mM-Hepes buffer, pH 7.5, containing 1 mM-DTSP and incubated for 15 min at 4°C as described (Laburthe *et al.*, 1984). The reaction was stopped by adding 500 μl of ice-cold 60 mM-Hepes buffer, pH 7.5, containing 60 mM-lysine as a reagent quench (Lomant & Fairbanks, 1976). The medium was centrifuged for 15 min at 20000g and the resulting pellet was suspended by five successive passages through a 25-gauge needle in 60 mM-Tris/HCl buffer, pH 6.8, containing 10% (v/v) glycerol, 0.001% (w/v) Bromophenol Blue and 3% (w/v) SDS. After heating for 30 min at 60°C , the suspension was centrifuged for 20 min at 40000g

and portions of the supernatant were applied to a 5–20% polyacrylamide slab gel (1.5 mm thick) according to the Laemmli (1970) method with a 3% stacking gel. The gels were run, fixed, stained and destained as described (Laburthe *et al.*, 1984). They were dried and exposed for 3–8 days at -80°C to a Trimax type XM film (3M) with a 3M Trimax intensifying screen. Autoradiographs were scanned at 600 nm using a spectrophotometer connected to an Omni-Scribe recorder (Houston Instruments).

Results

When rat liver plasma membranes are incubated with ^{125}I -VIP at equilibrium, washed free of unbound VIP and treated with 1 mM-DTSP, polyacrylamide-gel electrophoresis of membrane proteins reveals a major M_r 51 000 and a minor M_r 89 000 ^{125}I -VIP–protein complexes (Fig. 1, lane *a*). Several observations (not shown) indicate that the

complexes are produced by covalent cross-linking between ^{125}I -VIP and liver membrane proteins: (1) the bands are not detected if DTSP treatment is omitted; (2) the reagent quench lysine when added at 60 mM together with 1 mM-DTSP prevents the formation of the two bands; (3) reducing the disulphide bond of DTSP by incubation of DTSP-treated liver membranes with 0.7 M-2-mercaptoethanol for 30 min at 60°C results in the disappearance of the bands; (4) when albumin is omitted from the incubation medium of membranes, the two labelled complexes are unchanged, ruling out the possibility of the interference of albumin in the labelling pattern. The specificity of labelling of the M_r 51 000 and 89 000 components is tested by adding native VIP or other peptide hormones together with ^{125}I -VIP (Fig. 1). Native VIP (1 μM) completely abolishes the labelling of the two components (lane *b*) whereas the same concentration of cholecystokinin octapeptide (lane *c*) or glucagon (lane *d*) is without effect.

The labelling of M_r 51 000 and M_r 89 000 components is inhibited by the same range of VIP concentrations between 10^{-10} and 10^{-7} M as shown by microdensitometric scanning of autoradiographs (Fig. 2). The fine specificity of the M_r 51 000 and M_r 89 000 components is tested with two well-characterized VIP agonists: peptide histidine isoleucine and secretin (Bataille *et al.*, 1980; Laburthe *et al.*, 1983). Integration of peaks obtained by microdensitometric scanning of autoradiographs indicates that peptide histidine isoleucine and secretin are about 7 times and 200 times less potent than VIP, respectively, in inhibiting the covalent labelling of the two components (Fig. 3).

The guanine nucleotide GTP was shown previously to regulate the specific binding of ^{125}I -VIP to rat liver plasma membranes (Desbuquois, 1974; Amiranoff *et al.*, 1980b). We have thus tested its effect on the covalent labelling of the M_r 51 000 and M_r 89 000 components (Fig. 4). It appears that GTP in the 10^{-7} – 10^{-3} M concentration range reduces markedly their labelling, with a slightly higher potency for the M_r 89 000 component.

The pattern of specific labelling of rat liver membrane proteins by ^{125}I -VIP is not identical with that previously described for rat intestinal epithelial membranes. We have thus compared, within the same experiment and using exactly the same experimental procedure, the two patterns of labelling (Fig. 5). It appears clearly that the apparent M_r values of ^{125}I -VIP–binding site complexes in liver, i.e. 89 000 and 51 000 (Fig. 5, lane *a*), differ consistently from those observed in intestinal epithelium (Fig. 5, lane *c*) i.e. 76 000 for the high-affinity receptor and 36 000 for a low-affinity binding site (Laburthe *et al.*, 1984). The different pattern of labelling of membrane proteins

Fig. 1. Hormone specificity of covalent labelling by ^{125}I -VIP of liver membrane proteins as seen by SDS/polyacrylamide-gel electrophoresis

Conditions are as described under 'Experimental'. Membranes were incubated with ^{125}I -VIP and other hormones, then with 1 mM-DTSP. (a), The autoradiographic pattern of membranes incubated with ^{125}I -VIP alone; (b), with 1 μM -VIP; (c), with 1 μM -cholecystokinin octapeptide; (d), with 1 μM -glucagon.

Fig. 2. Dose-response of VIP in inhibiting the covalent labelling by ^{125}I -VIP of liver membrane proteins as seen by scanning of an autoradiograph

Conditions are as described under 'Experimental'. The mobility of proteins in the gel is expressed relatively to the mobility of the dye front. Native VIP concentrations are as follows: (a), ^{125}I -VIP alone; (b), 10^{-11}M -VIP; (c), 10^{-10}M -VIP; (d), $3 \times 10^{-10}\text{M}$ -VIP; (e), 10^{-9}M -VIP; (f), 10^{-8}M -VIP; (g), 10^{-7}M -VIP.

in the two tissues is not due to a difference in the purity of the membrane preparations since crude (Fig. 5, lane e) and purified (Fig. 5, lane a) liver membranes give exactly the same pattern of labelling.

Discussion

The reported data indicate that the cross-linking reagent DTSP promotes the covalent attachment of ^{125}I -VIP to proteins of rat liver plasma membranes. Polyacrylamide-gel electrophoresis of liver membrane proteins reveals that two high M_r components are specifically labelled, a major M_r 51 000 one and a minor M_r 89 000 one. Assuming one molecule of ^{125}I -VIP is bound/molecule of protein, the average M_r values of the membrane binding proteins that are covalently linked to VIP are 48 000 and 86 000. Besides these components, a considerable amount of radioactivity migrates at the dye front where ^{125}I -VIP migrates (see Fig. 1). This suggests that only a part of the reversible ^{125}I -VIP-binding site complexes has been covalently linked by DTSP treatment. Similar conclusions were drawn for the covalent labelling of intestinal VIP receptors using DTSP (Laburthe *et al.*, 1984) and in general for the cross-linking of peptide hormones to their receptors with hydroxy-succinimide esters (Pilch & Czech, 1979; Rebois *et al.*, 1981; Paglin & Jamieson, 1982; Donner, 1983).

The major M_r 48 000 and the minor M_r 86 000

Fig. 3. Dose-response of VIP and VIP agonists in inhibiting the covalent labelling of M_r 89 000 (left) and M_r 51 000 (right) ^{125}I -VIP-protein complexes

Conditions are as described under 'Experimental'. Membranes were incubated with ^{125}I -VIP and various concentrations of native VIP or VIP agonists, then treated with 1 mM-DTSP. The peak areas corresponding to the M_r 89 000 and M_r 51 000 ^{125}I -VIP-protein complexes were integrated after scanning of the autoradiographs. Results are expressed as a percentage of the initial peak area for membranes incubated with ^{125}I -VIP alone. Each point is the mean of three experiments. Standard errors were always below 13% of mean values. Abbreviation used: PHI, peptide histidine isoleucine.

Fig. 4. Dose-response of GTP in inhibiting the covalent labelling of the M_r 51000 and M_r 89000 ^{125}I -VIP-protein complexes

Conditions are as described under 'Experimental'. Membranes were incubated with ^{125}I -VIP and various concentrations of GTP, then treated with 1 mM-DTSP. The peak areas corresponding to the M_r 51000 (○) and M_r 89000 (●) ^{125}I -VIP-protein complexes were integrated after scanning the autoradiographs. Results are expressed as a percentage of the initial peak area for membranes incubated with ^{125}I -VIP alone. Each point is the mean $\pm$ S.E.M. of three experiments.

components of liver membranes that are labelled by ^{125}I -VIP display several properties expected for compounds of the specific VIP binding site. Their labelling is inhibited by low native VIP concentrations (see Figs. 2 and 3) that compare well with the concentrations needed to saturate VIP receptors in plasma membranes (Bataille *et al.*, 1974; Desbuquois, 1974) or isolated cells (Laburthe *et al.*, 1978a) from rat liver. Moreover, the ability of secretin and peptide histidine isoleucine to reduce the covalent labelling of the two components is consistent with the low affinity of these peptides for VIP binding sites in liver (Bataille *et al.*, 1980) as well as other tissues (Laburthe *et al.*, 1983). Taken together these results indicate that the two rat liver membrane proteins that link ^{125}I -VIP may contain the structural domain(s) necessary for recognizing VIP with a high affinity and for discriminating among VIP-related peptides. The attenuation by GTP of their labelling (see Fig. 4) suggests moreover that these two components are functionally coupled to a GTP regulatory protein within the liver plasma membranes before DTSP treatment. This is in good agreement with previous reports showing that the guanine nucleotide

Fig. 5. Pattern of covalent labelling by ^{125}I -VIP of liver membranes and intestinal membrane proteins as seen by SDS/polyacrylamide-gel electrophoresis

Conditions are described under 'Experimental'. Purified liver plasma membranes, intestinal plasma membranes and crude liver membranes (see the Experimental section) were incubated with ^{125}I -VIP, then treated with 1 mM-DTSP. (a, c, e), The autoradiographic pattern of liver plasma membranes, intestinal plasma membranes, and crude liver membranes incubated with ^{125}I -VIP alone, respectively; (b, d, f), with 10^{-7}M native VIP, respectively.

inhibits ^{125}I -VIP binding to liver membranes by enhancing the rate of dissociation of VIP-binding site complexes (Desbuquois, 1974; Amiranoff *et al.*, 1980b).

The presence of a minor M_r 86000 component with functional properties very similar to those of the major M_r 48000 binding component of rat liver plasma membranes raises the problem of their relationship. This molecular heterogeneity of VIP binding components can be interpreted in different ways: (1) the proteolytic degradation of one protein binding site during membrane preparation or incubation; (2) the cross-linking of one binding component with another protein species within the plasma membranes; (3) the existence of two distinct VIP-binding sites. The first hypothesis is unlikely for several reasons. (a) The ratio of labelling of the two components is identical in plasma membranes purified by the method of Neville (1968) and in crude plasma membranes (see the Experimental section and Fig. 5), although EDTA, an inhibitor of metal-dependent proteolytic enzymes, is present in the latter preparation and absent from the former one. (b) The incubation medium of membranes contains a proteinase inhibitor, bacitracin, at high concentration. Fur-

thermore, when binding is also performed in the presence of the potent inhibitor of proteolysis phenylmethanesulphonyl fluoride (results not shown), no modification of the pattern of labelling is observed. These observations contrast markedly with other systems such as, for example, the β -adrenergic receptors of turkey erythrocytes, where the ratio of photolabelling of the intact receptor and its degradation product are found to be very dependent on the method of tissue preparation and the presence of proteolysis inhibitors (Benovic *et al.*, 1983; Sibley *et al.*, 1984). The second hypothesis, i.e. the possibility that the minor M_r 86000 component results from the covalent cross-linking of a small proportion of the M_r 48000 binding site component to a neighbouring polypeptide within the plasma membrane, may be considered but with several restrictions. Indeed, cross-linking with succinimide esters under the low temperature conditions used in this study leads to very few protein aggregates (Pilch & Czech, 1979). Furthermore, few pre-existing complexes of major membrane proteins appear to exist at low temperature, and random collisions which can be detected by cross-linking occur only at higher temperatures (Peters & Richards, 1977). Finally, DTSP has a 1.2 nm span (Lomant & Fairbanks, 1976) and since random collisions are unlikely, it can cross-link only proteins that closely interact within the plasma membrane. In that respect, a good candidate would be a GTP regulatory protein functionally associated with VIP binding sites. This is supported by the ability of GTP to reduce the covalent labelling of the M_r 48000 protein (see Fig. 4).

The pattern of specific labelling of rat liver plasma membrane proteins by ^{125}I -VIP described here differs markedly from that previously observed in rat intestinal epithelial membranes, where an M_r 73000 component was characterized as a specific high-affinity VIP receptor and an M_r 33000 component identified as a specific low-affinity binding site (Laburthe *et al.*, 1984). These striking differences in the patterns of protein labelling are observed within the same experiment using identical conditions for membrane incubation and the cross-linking reaction (see Fig. 5). In addition, an identical pattern of labelling is observed for highly purified and crude membranes (see Fig. 5), likely indicating that the different purity of the hepatic and intestinal membrane preparations may not account for the discrepancies observed between the two tissues. Thus, our results may reflect biologically significant differences in the structure of VIP binding sites in liver and intestine. Whether this structural difference affects the primary structure and/or the glycosylation of the receptor protein remains to be investigated.

What is clear is that the available data concerning the affinity and structural requirements of VIP binding sites in liver and intestine (Laburthe *et al.*, 1978a; Bataille *et al.*, 1980) did not reveal any functional differences affecting the peptide recognizing domain(s). It may thus be suggested that the differences identified by the cross-linking procedure involve the structural domain of VIP binding sites responsible for the coupling of target proteins within the plasma membrane of intestinal and liver cells. This would be consistent with the markedly different ability of VIP to mobilize the cyclic AMP production system in the two tissues (Laburthe *et al.*, 1978a; Amiranoff *et al.*, 1980b).

In conclusion, the present paper shows that a major M_r 48000 membrane protein is involved in the specific binding of VIP to liver membranes. It also provides evidence suggesting structural differences between VIP binding sites in liver and intestine. It is, however, clear that ultimate understanding of the nature of these differences will require the purification of individual components of VIP receptor/effector systems. When available, radiolabelled photoaffinity probes for VIP will probably permit a more precise analysis of the structural properties of VIP receptors.

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