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Properties of Vasoactive Intestinal Peptide-Receptor Interaction in Rat Liver Membranes

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The properties of the specific receptors for vasoactive intestinal peptide (VIP) in rat liver plasma membranes have been studied by using ^{125}I-VIP as a tracer. The binding of the peptide was a reversible, saturable and specific process, as well as time and temperature dependent. Peptide inactivation was also dependent on time and temperature and remained relatively low in the standard conditions used, as it happened in the inactivation of the binding sites.

The binding data were compatible with the existence of two classes of VIP receptors: a high affinity ($K_d = 4.2 \times 10^{-10}$ M) and low binding capacity (1.5 pmol VIP/mg protein) class and another one of low affinity ($K_d = 1.7 \times 10^{-7}$ M) and high binding capacity (38.6 pmol VIP/mg protein). The specificity of the binding sites for VIP was established from the fact that binding of ^{125}I-VIP was inhibited by native VIP and by 60-fold higher concentrations of secretin but not by the parent hormone glucagon, by insulin or somatostatin at concentrations as high as 10^{-6} M.

Vasoactive intestinal peptide (VIP), first isolated from porcine gut (22), is actually considered as a possible neurotransmitter or neuromodulator produced by VIPergic nerves and released by nerve endings at its numerous sites of action (see refs. 9, 12, 21 for review). Membrane VIP receptors coupled to adenylate cyclase have been demonstrated in cells and/or plasma membranes from liver (3, 6, 11), exocrine pancreas (5, 15, 19), adipose tissue (3), intestinal epithelium (1, 2, 13, 17, 18) and brain (7, 20, 25).

Among the great number of biological actions attributed to VIP, this peptide possesses glycogenolytic effects in liver (10, 22). Published data on liver VIP receptors are scarce (3, 6, 11) and there is only a report (6) that describes in some detail their characteristics; however, that

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work (6) does not show the existence of two distinct classes of VIP receptors, a common feature demonstrated in many other systems (2, 5, 18, 20) and parameters such as inactivation of binding sites were not studied.

The present investigation was undertaken in order to define the characteristics of VIP receptors in rat liver membranes with regard to the kinetics, stoichiometry and specificity of the binding process.

Materials and Methods

Female Wistar rats weighing between 150-200 g were used. Liver plasma membranes were isolated according to NEVILLE (16). The partially purified preparation (step 11 in the reference 16) was obtained and stored at —80° C until used. As an index of plasma membrane purification, 5′-mononucleotidase activity was measured (26); a purification factor of about 10-fold was obtained. Protein was measured by the method of LOWRY *et al.* (14) using bovine serum albumin (BSA) as standard.

^{125}I-labelled VIP (^{125}I-VIP) was prepared at a specific activity of about 250 Ci/g and possessed binding properties identical to those of native VIP (18).

The binding assay was conducted in a standard incubation solution (500 μl final volume) consisting of 50 mM Tris-HCl (pH 7.5), 2 mg/ml BSA, 1 mg/ml bacitracin and 45 pM ^{125}I-VIP in the absence or in the presence of native VIP up to 10^{-6} M. Reaction was started with the addition of membranes at 50 μg protein/ml. Unless otherwise indicated, incubations were performed at 15° C for 90 min. Membrane-bound peptide was separated by centrifugation at 10,000×*g*, as previously described (18). Results are expressed as specific binding; this is obtained by subtracting from the total bound that amount of ^{125}I-VIP which is not displaced by an excess (10 μM) of native peptide. Nonspecific binding represented about 3-4 % of the total radioactivity added. Each individual experiment was performed in triplicate.

The inactivation of ^{125}I-VIP in the incubation medium after exposure to membranes was studied by the ability of the peptide to rebind to fresh membranes (18). The inactivation of binding sites was measured by the loss of ability of membranes to bind tracer after preincubation in the absence of peptide. Dissociation of the labelled peptide-membrane complex was performed by the addition of an excess of native VIP (1 μM) at a steady state.

Synthetic VIP corresponding to the porcine peptide and synthetic somatostatin (cyclic form) were purchased from Peninsula Laboratories, secretin from Kabi, insulin and glucagon from Novo, bacitracin and BSA from Sigma, and carried-free Na^{125}I (IMS 30) from Amersham. All other chemicals were reagent grade.

Results

VIP binding and inactivation: dependent on time, temperature, membrane concentration and pH. Specific ^{125}I-VIP binding to liver membranes was dependent on time and temperature (fig. 1, top). At 15° C the specific binding increased with time and reached a plateau after approximately 60 min and remained constant until 4 h incubation. Increasing the temperature to 30° C produced an acceleration in the rate of the binding reaction and a maximal amount of ^{125}I-VIP bound somewhat higher than at 15° C. The percentage of nonspecific binding was similar at both temperatures and remained constant throughout the incubation period.

As shown in figure 1 (bottom), the inactivation of ^{125}I-VIP in the medium after incubation with membranes increased with time and was also dependent on

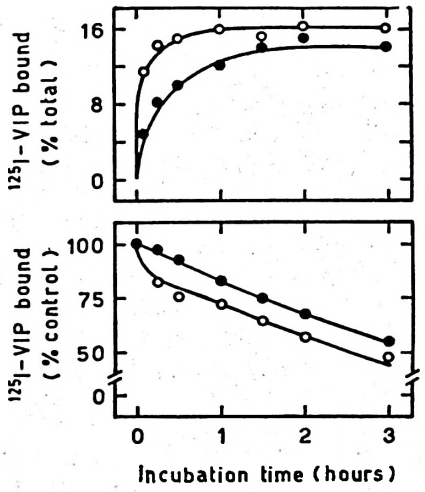

Fig. 1. *Time course of binding (top) and inactivation (bottom) of ^{125}I-VIP by liver membranes.*
Membranes were incubated with ^{125}I-VIP at 15° C or 30° C. Specific binding (top) and inactivation (bottom) of the labelled peptide were measured at the time intervals indicated. Each point is the mean of triplicates.

temperature. Inactivation of tracer was greater at 30° C than at 15° C suggesting that the differences between both patterns of binding were higher than those reflected by the association experiments. In subsequent experiments, incubations were performed at 15° C for 90 min, i.e. when an apparent steady-state was observed (about 15 % ^{125}I-VIP specifically bound) and the inactivation of the peptide was relatively low (about 25 %).

The binding of ^{125}I-VIP depended on the membrane concentration (fig. 2, top). It was linearly related to membrane protein concentration up to 150 µg/ml but at higher concentrations the observed binding was less than expected, probably because of the great extent of peptide inactivation (fig. 2, bottom). Further incubations were then performed with 50 µg membrane protein/ml.

The binding of ^{125}I-VIP to liver membranes occurred over a relatively large range of pH (fig. 3, top). Maximal binding was observed in the 7-8 range of pH so that a pH of 7.5 was chosen as optimal to study the characteristics of liver VIP receptors.

Inactivation of binding sites. This condition is generally neglected in the study of peptide-receptor interaction in spite of its importance when different physiological states are correlated with a change in the number of receptors. Preincubation of liver membranes in a medium devoid of peptide resulted in a progressive decrease of the ability to bind ^{125}I-VIP due to an inactivation or a shedding of the receptors (fig. 3, bottom). The percentage of binding of ^{125}I-VIP after 90 min preincubation decreased by about 30 % with respect to control values.

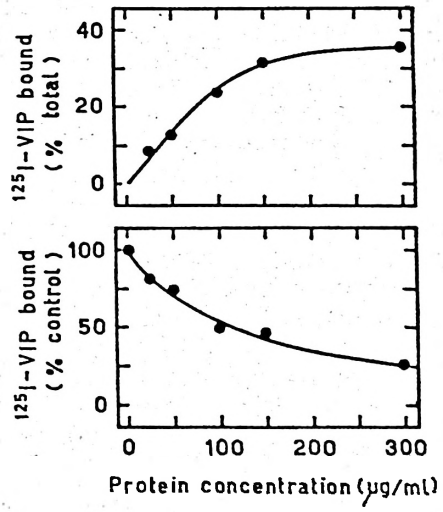

Fig. 2. *Binding (top) and inactivation (bottom) of ^{125}I-VIP as a function of membrane concentration.*
Increasing membrane concentrations were incubated with ^{125}I-VIP at 15° C. Specific binding (top) and inactiavtion (bottom) of the labelled peptide were measured after 90 min incubation. Each point is the mean of triplicates.

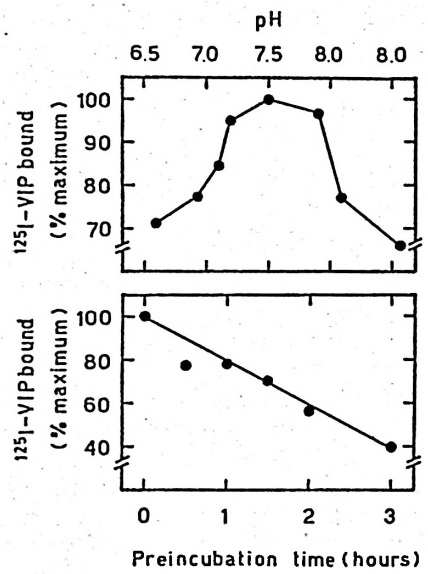

Fig. 3. *Dependence of binding on pH (top) and inactivation of binding sites (bottom).* Top: membranes and ^{125}I-VIP were incubated for 90 min at 15° C in the range of pH 6.5-8.5. Bottom: membranes were preincubated at 15° C for the time intervals indicated; then ^{125}I-VIP was added and the incubation continued for 90 min at 15° C. Each point represents the specific binding as the mean of triplicates.

Dissociation studies. The dissociation of peptide from the receptors was studied in experiments in which membranes were preincubated with ^{125}I-VIP (fig. 4). The addition of an excess of native VIP to the tracer-receptor complex at steady-state induced a time-dependent dissociation of ^{125}I-VIP from membranes. The pattern of dissociation did not follow a monoexponential time course, indicating the existence of more than one class of binding sites. Assuming two classes of binding sites with different affinities, the dissociation of bound ^{125}I-VIP can be considered as the sum of two first-order processes with apparent half-times of dissociation of about 25 and 215 min for the low and for the high-affinity sites, respectively.

Binding at steady-state. Liver membranes were incubated during 90 min with ^{125}I-VIP and increasing concentrations of native peptide in order to determine the dissociation constant (K_d) for the binding of VIP (fig. 5). Assuming that the peptide reacted with one single class of receptors, half-maximal inhibition of tracer binding was observed at about 4.8×10^{-10} M VIP. However, when analyzed with the Scatchard plot (23) the data could not be fitted by a single straight line, but showed a curvilinear graph (fig. 5, inset). Among the theoretical models compatible with such data (4) two are usually considered in the literature: binding sites heterogeneity or peptide-dependent negative cooperativity among receptors. Negative coopera-

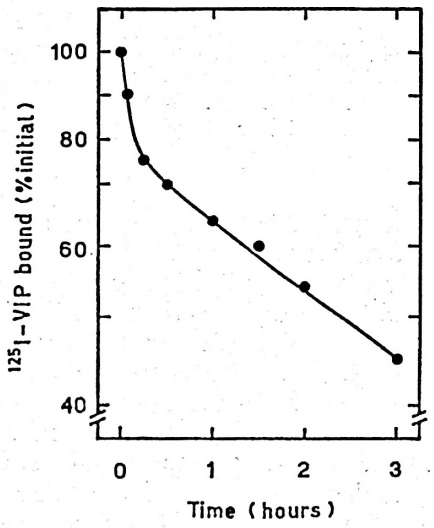

Fig. 4. *Dissociation of ^{125}I-VIP from the receptors.* Membranes and ^{125}I-VIP were incubated for 90 min at 15° C. The complex labelled peptide-membranes was separated by centrifugation and the supernatant discarded and replaced by an identical volume of fresh medium in the presence of 1 μM native VIP. Specific binding was then determined at various time intervals of further incubation at 15° C. Each point represents the specific binding as the mean of triplicates.

tivity was not tested experimentally due to the difficulties from using plasma membranes instead of cells (24). Assuming the existence of two classes of binding sites, as suggested by Scatchard plot and dissociation experiments, it was calculated that the high affinity site has a K_d of 4.2×10^{-10} M and a binding capacity of 1.5 pmol VIP/mg protein whereas the low affinity site has a K_d of 1.7×10^{-7} M and a binding capacity of 38.6 pmol VIP/mg protein. Therefore the high and the low affinity sites represent about 4 % and 96 % of the total number of receptors, respectively.

Specificity studies. To test the specificity of the VIP receptors, liver membranes were incubated with ^{125}I-VIP in the presence of various unlabelled peptides (fig. 6). Secretin, a hormone having

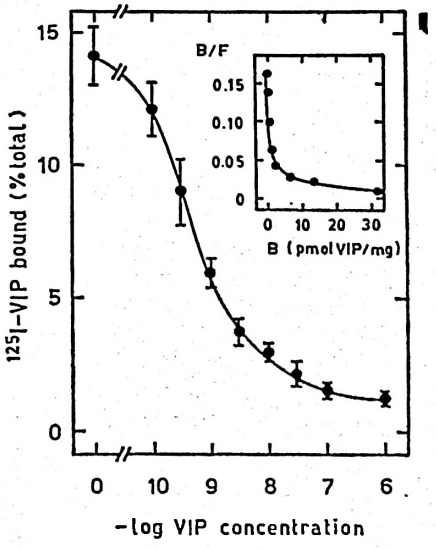

Fig. 5. *Competitive inhibition of ^{125}I-VIP binding by native peptide.*
Membranes and ^{125}I-VIP were incubated with increasing concentrations of unlabelled peptide for 90 min at 15° C. Each point represent the specific binding of ^{125}I-VIP as the mean ± S.E.M. of seven experiments performed in triplicate. Inset: Scatchard analysis (23) of the same data.

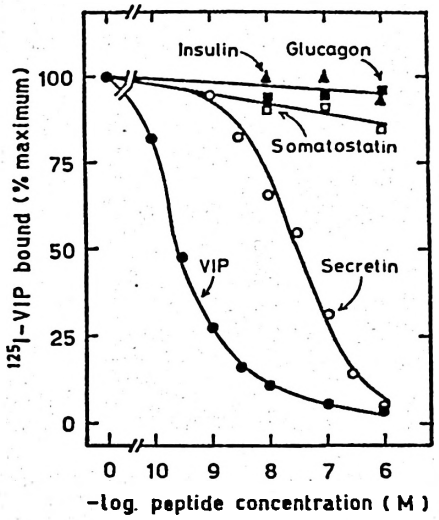

Fig. 6. *Competitive inhibition of ^{125}I-VIP binding by native peptide.*
Membranes and ^{125}I-VIP were incubated with native VIP, secretin, glucagon, insulin and somatostatin at the concentrations indicated. Results are expressed as the percentage of the radioactivity specifically bound in the absence of native peptide. Each point is the mean of triplicates.

9 amino acid residues in common with VIP, inhibited competitively the binding of the tracer in a parallel manner to that of VIP. The concentration of secretin that caused half-maximal inhibition was 3×10^{-8} M and about 60 times as much secretin was required to exert the same effect as VIP. Glucagon is another peptide hormone structurally related to VIP but it did not inhibit the binding of ^{125}I-VIP with the receptor up to concentrations as high as 10^{-6} M. A similar absence of effects on VIP binding was observed with another unrelated peptides such as insulin and somatostatin.

Discussion

Although VIP is known to bind to liver cells. (11) and membranes (3, 6), the

physical characteristics of this interaction have not been extensively studied. The present investigation shows a detailed study of the properties of VIP receptors in rat liver plasma membranes: kinetics of association and dissociation, stoichiometry and specificity.

The binding sites exhibited many charactiristics common to other receptor systems such as dependence of the interaction on time, temperature and pH, reversibility, saturability and specificity, in agreement with previous reports in the literature on VIP binding to liver (6), fat (3), brain (20, 25), intestinal epithelial (2) and pancreatic acinar (15) membranes.

Inactivation of VIP during its interaction with the membranes increased with time and temperature but it could be substantially reduced by performing the incubations at 15° C and at low concentration of protein cell membranes, in accordance with the observations in other systems (2, 5, 18). A certain extent of binding sites inactivation (or release of the receptors into the incubation medium) could be observed. Inactivation of both VIP and receptors are two factors that modify the validity of the calculated K_d and binding capacity and, for this reason, these data can only be considered as apparent values.

When VIP binding to liver membranes is considered to be mediated by a single class of receptors, the calculated K_d of 4.8×10^{-10} M is in close agreement with that reported by DESBUQUOIS (6) in a similar system ($K_d = 1.6 \times 10^{-10}$ M). Such is not the case with respect to the total binding capacity of about 40 pmol VIP bound/mg protein in the present investigation and 0.8 pmol VIP bound/mg protein in that previous paper (5), probably due to differences in the purification methods of rat liver membranes.

The saturation curve of VIP binding was curvilinear with an upward concavity when represented according to SCATCHARD (23), by analogy to other VIP-receptor systems so far characterized (5, 11, 18). The possibility of negative cooperativity depending on the participation of a mobile receptor to a peptide-receptor-effector complex or on site-site interactions among receptors is unlikely. The stoichiometric data have then been interpreted as representing two classes of VIP receptors: one class with high affinity and low binding and another class with low affinity and high binding capacity. Furthermore, this pattern of receptor heterogeneity is in agreement with the multiorder kinetics of dissociation of the peptide-receptor complex. The existence of high affinity sites could also be attributed to the low concentration of ^{125}I-VIP (45 pM) that was able to bind to the membrane preparation.

The biological significance of a class of low affinity receptors has been questioned frequently in the literature; in fact, this class of sites is often considered as irrelevant since their occupancy requires very high concentrations of peptide. However, it must be considered that VIP is locally liberated by nerve terminals (2, 3, 4) in high enough quantities to recognize these sites. The reported values of K_d and binding capacity for the two classes of receptors are of the same order as those found in various systems of VIP binding to plasma membranes (11, 18) but differ from others (20) in which the affinities of the binding sites were lower probably due to the presence of VIP in the membrane preparation used. Assuming a value of 2×10^7 liver cells/mg of liver plasma membrane protein (8) it can be deduced from present data that there are about 4.6×10^4 high affinity and 1.1×10^6 low affinity VIP binding sites in liver membranes; these values are nearly identical to those found in hepatocytes (11) and enterocytes (18).

Among the various peptides tested, only VIP and secretin inhibited the binding of ^{125}I-VIP to liver membranes whereas glucagon, insulin and somatostatin did

not. The parallelism between the curves of displacement for VIP and secretin indicates that these two peptides acted through the same set of receptors but secretin was 60-fold less effective than VIP. The fact that liver VIP receptors possessed a low affinity for secretin and did not recognize the parent hormone glucagon is a common feature to all the VIP binding systems so far characterized (2, 5, 6, 7, 12, 20, 25).

Resumen

Se han estudiado las propiedades de los receptores específicos para el péptido intestinal vasoactivo (VIP) en membranas hepáticas de rata utilizando I^{125}-VIP como trazador. La unión del péptido fue un proceso dependiente del tiempo y de la temperatura, reversible, saturable y específico. Su inactivación dependió también del tiempo y de la temperatura y permaneció relativamente baja en las condiciones standard utilizadas, así como la inactivición de los receptores.

Los datos fueron compatibles con la existencia de dos clases de receptores de VIP: una clase de alta afinidad ($K_d = 4{,}2 \times 10^{-10}$ M) y baja capacidad (1,5 pmol VIP/mg proteína) y otra clase de baja afinidad ($K_d = 1{,}7 \times 10^{-7}$ M) y alta capacidad (38,6 pmol VIP/mg proteína). La especificidad de los receptores de VIP se demostró por el hecho de que sólo el VIP nativo y concentraciones 60 veces superiores de secretina inhibieron la unión de I^{125}-VIP, lo que no ocurrió con la hormona estructuralmente relacionada con glucagón ni con insulina o somatostatina a concentraciones tan elevadas como 10^{-6} M.

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