Characterisation of a high-affinity VIP receptor in human lung parenchyma

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Received 6 January 1986

A method is described for preparing human lung parenchymal membranes essentially free of carbon contamination. Using this technique, a high-affinity ^{125}I-VIP-binding site has been characterised. The receptor density is approx. 200 fmol/mg protein, and the K_d of ^{125}I-VIP by saturation binding is 200 pM. The dissociation kinetics are complex and cannot be described by first-order kinetics. Several VIP-related peptides displace ^{125}I-VIP from this binding site with a rank order of potency: VIP > rat GRF > PHM > PHI > human GRF > secretin > glucagon. Displacement curves of these peptides exhibited slope factors significantly less than unity with the exception of human GRF.

VIP receptor (Human lung) Radioligand binding

1. INTRODUCTION

VIP is a potent relaxant of mammalian tracheobronchial and pulmonary smooth muscle, both in vivo [1–3] and in vitro [4,5]. Dense VIP-ergic innervation of both airways and pulmonary vessels has been demonstrated in several species including man [6,7]. VIP receptors have also been described in lung parenchyma in rat and guinea pig using radioligand-binding techniques [8]. Although the presence of similar receptors has been inferred for human lung because of bronchodilator responses in vitro and in vivo and also from adenylate cyclase stimulation experiments [8,9], such receptors have hitherto not been demonstrated by binding studies. The main reason has been the excessively high level of non-specific binding [8] due to contamination of adult human lung by carbon particles. We describe a technique for overcoming this problem and we have been able to characterise a high-affinity ^{125}I-VIP-binding site in human lung parenchyma using a filtration assay system.

Abbreviations: VIP, vasoactive intestinal peptide; PHI, peptide histidine isoleucine; PHM, peptide histidine methionine; PMSF, phenylmethylsulphonyl fluoride; GRF, growth hormone-releasing factor

2. MATERIALS AND METHODS

2.1. *Materials*

^{125}I-VIP was obtained from Amersham International (Amersham, England); unlabelled VIP, secretin, PHI, glucagon, Tris, bovine serum albumin, polyethyleneimine, leupeptin and soya bean trypsin inhibitor from Sigma (Poole, England); PHM, rat GRF and human GRF from Peninsula Laboratories (St. Helens, England); Lymphoprep from Nyegaard and Sepharose CL-4B from Pharmacia. Other chemicals were of Analar grade from British Drug Houses.

2.2. *Methods*

Human lung was obtained at resection or from post-mortem specimens obtained within 12 h of death. Tissue was rapidly frozen in liquid nitrogen and then stored at −70°C. Approx. 20–30 g lung were used at a time for membrane preparation. Frozen tissue was placed in cold buffer containing

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00145793/86/$3.50

10 mM Tris, 0.25 M sucrose, 5 mM EDTA, 0.1 mM PMSF, 20 μg/ml soya bean trypsin inhibitor and 10 μg/ml leupeptin (pH 7.4). After thawing the tissue was minced as finely as possible with scissors and then homogenised using a Polytron (setting 10, 30 s × 4). The homogenate was centrifuged at 1000 × *g* for 15 min, and the supernatant filtered through two layers of cheesecloth. The filtrate was centrifuged at 30000 × *g* for 15 min and the pellet resuspended in Tris/sucrose buffer containing 5 mM EDTA and 0.1 mM PMSF (approx. 1 ml buffer per 2 g frozen wt). The membrane suspension was layered onto Lymphoprep (3.5 ml suspension: 1.5 ml Lymphoprep) and centrifuged at 100000 × *g* for 1 h. Membranes were collected at the interface and diluted 1:2 with buffer containing 25 mM Tris and 5 mM $MgCl_2$, pH 7.4, and centrifuged at 45000 × *g* for 15 min. The pellet was resuspended in the same buffer and mixed with Sepharose CL-4B gel (9:1, v/v) previously washed twice in buffer. The mixture was centrifuged at 2000 × *g* for 4 min and the supernatant, consisting of gel-free membrane suspension, was carefully removed. The protein content of the suspension was assayed by the method of Bradford [10] and the suspension diluted to a protein concentration of 500–1000 μg/ml. The final preparation was divided into aliquots and stored at −70°C.

In saturation binding assays, 20 μl membrane suspension were incubated with 10 μl ^{125}I-VIP (final concentration 0.125–1.25 nM) and 10 μl buffer or unlabelled VIP (final concentration 1 μM) to define non-specific binding. The incubation buffer consisted of 25 mM Tris, 5 mM $MgCl_2$, 0.5% bovine serum albumin, 0.1% bacitracin. Incubation time was usually 15 min at 37°C. The incubation was terminated by rapid filtration under reduced pressure through Whatman GF/C filters. The filters were washed 3 times with 4 ml ice-cold buffer containing 50 mM Tris, 0.5 mM EDTA, 0.2% BSA (pH 7.4). To reduce ligand binding to filters, the latter were pre-soaked in 0.3% polyethyleneimine. Without this precaution over

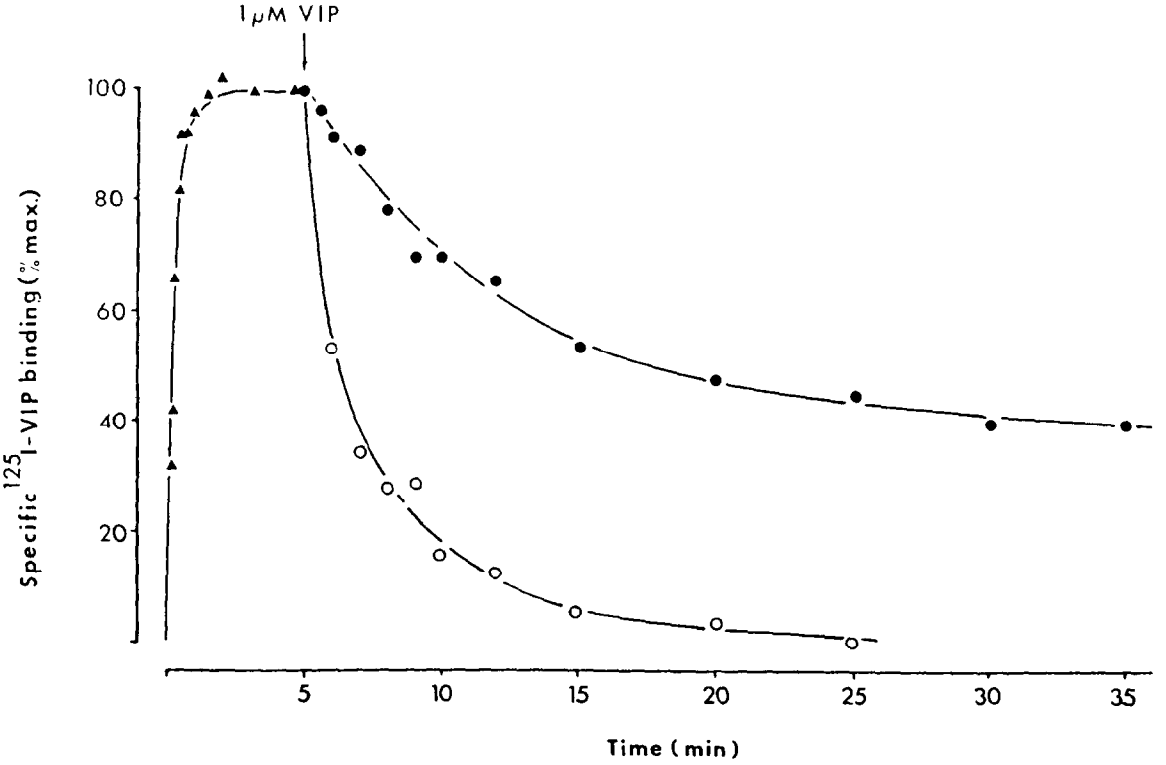

Fig.1. Association and dissociation kinetics of ^{125}I-VIP (0.25 nM) binding to lung membranes in the presence (●) and absence (○) of 100 μM GTP.

90% of ^{125}I-VIP was bound to filters in the absence of membrane. After filtration, the filter discs were dried and counted in a Packard Multiprias gamma spectrometer. In kinetic and displacement experiments the ^{125}I-VIP concentration was 0.25 nM. Experimental conditions were otherwise as described.

In saturation experiments binding parameters were derived using Scatchard analysis [11]. IC_{50} values in displacement experiments were obtained by plotting normalised data on probit paper followed by line fitting using linear regression. K_i values were calculated according to the equation of Cheng and Prusoff [12].

3. RESULTS

At 37°C there is very rapid binding of ^{125}I-VIP to lung parenchymal membranes. Binding reached steady state within 2 min (fig.1). After the addition of 1 μM VIP, in the absence of GTP, dissociation was not complete and could not be described by first-order kinetics. Under these conditions, the dissociation curve can be fitted to two components with K_{-1} values of 0.178^{-1} and 0.01^{-1}, respectively. In the presence of 100 μM GTP, first-order dissociation kinetics could be applied with a K_{-1} of 0.311^{-1}.

Table 1

Inhibition of ^{125}I-VIP binding to human lung membranes: K_i values and Hill slope factors (n_H)

	K_i (nM)	n_H
VIP	0.67 ± 0.19	0.65 ± 0.03
PHM	27.0 ± 26.2	0.69 ± 0.01
PHI	84.0 ± 26.2	0.64 ± 0.03
rGRF	26.3 ± 6.2	0.81 ± 0.08
hGRF	384 ± 23	0.96 ± 0.08
Secretin	1852 ± 431	0.73 ± 0.08
Glucagon	⩾100000	–

^{125}I-VIP concentration: 250 pM for all experiments; values are means ± SE; n = 3–6

Over the concentration range studied, Scatchard analysis describes a single population of binding sites (fig.2). Receptor density is approx. 200 fmol/mg protein with a K_d of 200 pM. If the ligand concentration is increased to more than 2 nM the Scatchard plot becomes curvilinear, suggesting the presence of a second binding site of lower affinity (not shown).

Displacement curves for several VIP-related peptides are shown in fig.3, and the K_i values and Hill factors are summarised in table 1. Peptides generally displace from a smaller number of sites

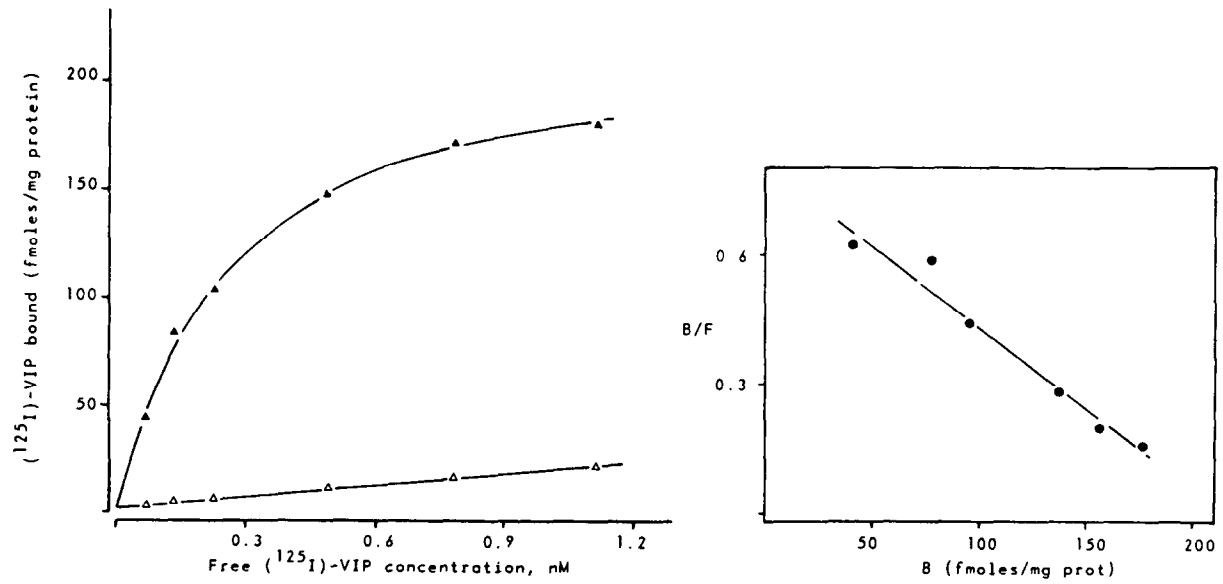

Fig.2. Typical saturation binding isotherm of specific ^{125}I-VIP binding to lung membranes (n = 3, lungs from different individuals). B_{max} range 183–203 fmol/mg protein. K_d range 0.16–0.25 nM. (Inset) Scatchard plot.

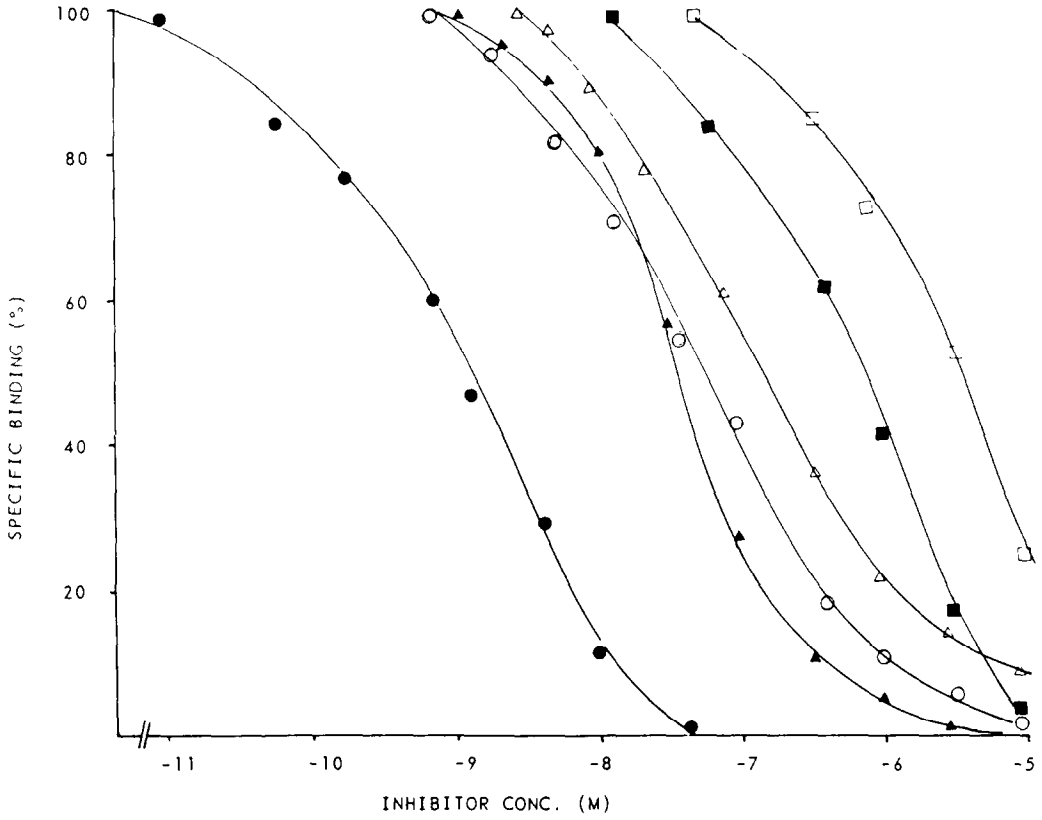

Fig.3. Inhibition of ^{125}I-VIP (0.25 nM) binding by unlabelled VIP (●), PHM (○), PHI (△), rat GRF (▲), human GRF (■) and secretin (□). Specific binding is defined by 10^{-6} M VIP. Typical curves from n = 3–6.

than unlabelled VIP itself, a common finding when the same compound is used as both the labelled and the displacing ligand. Only in one case, human GRF, does the slope factor approximate to unity. Other peptides have slope factors of about 0.7 although the displacement curve for that of GRF is somewhat steeper (n_H = 0.81).

4. DISCUSSION

Using the techniques described we have been able to obtain consistently low levels of non-specific binding, usually 10% or less at 250 pM ^{125}I-VIP. Binding of ^{125}I-VIP is saturable under the conditions noted. Although we have not made any attempt to quantitate the residual carbon contamination, these results indicate that it is of minimal significance. Preparations which did not include the Sepharose purification step were often unsatisfactory in that excessive contamination usually remained, as judged by high non-specific binding and lack of saturability. The very low level of filter-bound ligand also contributes to the favourable total/non-specific ratio.

We have yet to prepare animal lung membranes using an identical protocol, but the density of VIP-binding sites in human lung parenchyma is clearly less than in rodent species hitherto studied (e.g. guinea pig), where receptor density is about 1 pmol/mg protein (Dickinson, in preparation).

Studies in other species have suggested that there may be a second VIP-binding site in the lung with considerably lower affinity (K_d about 20–25 nM) [13]. We have not attempted to study this site in man, although adenylate cyclase stimulation studies suggest that it exists [14]. The shallow displacement curves for unlabelled VIP and other related peptides may reflect the presence of such a site, but could be anticipated in any case when agonists are used as both radioligands and com-

peting agents. The pharmacological profile of the site described resembles that in other mammalian lungs, though there are some interesting differences. We have been able to use a wider range of peptides than hitherto described in lung VIP receptor experiments, and there is an intriguing possibility that at least one of these peptides (human GRF) might be an antagonist, though not a very potent one. At present we have no functional evidence for this, but if it were confirmed it would provide a useful tool for the study of the VIP receptor in lung and possibly in other tissues. Human GRF may then serve as the starting point for the development of structural analogues which may be more potent antagonists.

Another interesting observation relates to the kinetic experiments. As previously mentioned, the dissociation curve in the presence of excess unlabelled VIP cannot adequately be described in terms of first-order kinetics. The data suggest that there may be a binding site of very high affinity in addition to that described in detail above. Kinetic data of this kind have been described for other VIP receptors [15–17], and also for insulin [18], but an adequate explanation has not yet been proposed. No functional correlate for such a site has been described in the lung, but in blood vessels VIP is a very potent dilator with effective concentrations in the picomolar range. It has recently been suggested that at these low concentrations the vasodilator effect of VIP is not mediated by adenylate cyclase activation [19].

In conclusion, we have characterised a putative high-affinity VIP receptor in human lung parenchymal membranes. The pharmacological characteristics of this receptor largely resemble those described in other mammalian lung VIP receptors. The membrane preparation technique may also be applicable to binding studies with other ligands, particularly in human lung, and the filtration assay may prove quicker and more convenient than the centrifugation techniques usually used in VIP-binding experiments.

ACKNOWLEDGEMENT

This work was partly supported by a grant from the Asthma Research Council.

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