Characterization of functional receptors for vasoactive intestinal peptide (VIP) in rat peritoneal macrophages

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(Received 2 August; revised version received 7 December 1990; accepted 16 January 1991)

Key words: VIP receptor; Rat peritoneal macrophage; cyclic AMP production

Summary

Functional vasoactive intestinal peptide (VIP) receptors have been characterized in rat peritoneal macrophages. The binding depended on time, temperature and pH, and was reversible, saturable and specific. Scatchard analysis of binding data suggested the presence of two classes of binding sites: a class with high affinity ($k_d = 1.1 \pm 0.1$ nM) and low capacity (11.1 ± 1.5 fmol/10⁶ cells), and a class with low affinity ($k_d = 71.6 \pm 10.2$ nM) and high capacity (419.0 ± 80.0 fmol/10⁶ cells). Structural requirements of these receptors were studied with peptides structurally or not structurally related to VIP. Several peptides inhibited $^{125}$I-VIP binding to rat peritoneal macrophages with the following order of potency: VIP > rGRF > hGRF > PHI > secretin. Glucagon, insulin, somatostatin, pancreastatin and octapeptide of cholecystokinin (CCK 26–33) were ineffective. VIP induced an increase of cyclic AMP production. Half-maximal stimulation (ED₅₀) was observed at 1.2 ± 0.5 nM VIP, and maximal stimulation (3-fold above basal levels) was obtained between 0.1–1 μM. Properties of these binding sites strongly support the concept that VIP could behave as regulatory peptide on the macrophage function.
Introduction

Vasoactive intestinal peptide (VIP) is a 28 amino acid neuropeptide that has been first isolated from porcine duodenum [1]. VIP is now regarded as a member of the so-called secretin-glucagon family of polypeptides (secretin, glucagon, growth hormone releasing factor, helodermin, GIP, VIP and PHI/PHM-27) [2–4] and is considered as a local hormone or a neuromodulator released by VIP-containing nerves [5]. Experimental evidence is accumulating that this neuropeptide plays a role in communication between the nervous and immune systems [6–8]. In this context, specific receptors for VIP have been demonstrated on human peripheral blood mononuclear cells [9–11], human blood monocytes [12], mouse lymphocytes [13], rat lymphoid cells [14] and rabbit spleen lymphocytes [15]. On the other hand, VIP has been shown to activate adenylate cyclase in human lymphocytes membranes [16], to stimulate cyclic AMP accumulation and to activate cyclic AMP-dependent protein kinase in human peripheral blood mononuclear cells [9,11,17].

In this paper, we demonstrated for the first time the presence of functional VIP receptors with high affinity and specificity for VIP in rat peritoneal macrophages.

Materials and Methods

Chemicals

Synthetic rat VIP, porcine peptide histidine isoleucine amide (PHI), rat and human growth hormone releasing factors (rGRF and hGRF, respectively), octapeptide of cholecystokinin (CCK 26–33), porcine pancreastatin, secretin and somatostatin were purchased from Peninsula Laboratories Europe (Merseyside, U.K.); porcine glucagon and porcine monocomponent insulin from Novo (Copenhagen, Denmark); bacitracin, bovine serum albumin (BSA) (fraction V), 3-isobutyl-1-methyl-xanthine (IBMX) from Sigma (St. Louis, MO, U.S.A.); carrier-free Na125I (IMS 30, 100 mCi/ml) and cyclic AMP assay kits were obtained from Radiochemical Center (Amersham, U.K.). Synthetic rat VIP was radioiodinated by chloramine T method to a specific activity of about 800 Ci/mmol [9]. Purification of labeled tracer was performed on a Sephadex G-50 column (1 x 30 cm) eluted with 0.2 M acetic acid containing 0.5 ~ (w/v) BSA and 0.03 ~o (w/v) bacitracin. All other chemicals were reagent grade.

Preparation of peritoneal macrophages

Peritoneal macrophages were elicited from Wistar rats according to the Tsunawsky and Nathan method [18]. Utmost precautions were taken such that the animals remained free from infection by environmental pathogens. Briefly, female Wistar rats (body weight 250–300 g), allowed free access to food and water, were injected intraperitoneally 4 days before harvest with 5 ml of 6% sodium caseinate. Animals were killed by decapitation and, immediately, peritoneal cavity was washed with 2 x 10 ml of cold 0.15 M NaCl. Cells were pelleted by centrifugation, resuspended in 0.15 M NaCl and immediately used for experiments. Viability, as determined by trypan-blue exclusion, was always greater than 95%. Mean cells per rat varied from (20–30) x 10^6, of
which 85–95% were macrophages, 2–3% were lymphocytes and 7–9% were polymorphonuclear leukocytes by morphological criteria in Giemsa and Papanicolaou staining techniques [19].

**Binding studies**

In standard conditions, rat peritoneal macrophages (1.5 × 10⁶ cells/ml) were incubated at 15 °C in 0.5 ml of 35 mM Tris-HCl buffer (pH 7.5) containing 50 mM NaCl, 1.4% (w/v) bovine serum albumin, 1 mg/ml bacitracin and 45 pM ¹²⁵I-VIP either alone or together with increasing concentrations of unlabelled VIP (up to 0.1 μM) or other peptides. After 90 min incubation, cell-bound peptide was separated by centrifugation, as described previously [14], and the radioactivity associated with the cells was measured in a LKB gamma counter. Specific binding was calculated from total binding by subtracting nonspecific binding, as determined by binding of tracer in the presence of unlabelled VIP at 1 μM. Nonspecific binding was about 3–4% of the total radioactivity added. Each individual experiment was performed in triplicate. The degradation of

![Graph](image-url)

**Fig. 1.** Time-course of specific binding (top) and degradation (bottom) of ¹²⁵I-VIP by rat peritoneal macrophages as a function of temperature. ¹²⁵I-VIP (45 pM) was incubated with cells (1.5 × 10⁶ cells/ml) at 15 °C (■) or 30 °C (○). Specific binding of the labelled peptide (top) was determined at the indicated time intervals. At the same time periods, integrity of the labelled peptide remaining free in the incubation medium was also evaluated (bottom). Each point is the mean of triplicates. Two other separate experiments gave similar results.
**Cyclic AMP production**

Cyclic AMP was determined with minor modifications as previously described [11]. In a standard assay, rat peritoneal macrophages (10⁶ cells/ml) were incubated at 15 °C in 0.5 ml of 35 mM Tris-HCl buffer (pH 7.5) containing 50 mM NaCl, 1.4% (w/v) bovine serum albumin, 1 mg/ml bacitracin, 0.2 mM 3-isobutyl-1-methyl-xanthine (IBMX) in the absence or presence of increasing concentrations of unlabeled VIP (up to 0.1 μM). After 45 min incubation, the reaction was stopped by the addition of 2.5 ml methanol. The precipitate was removed by centrifugation, aliquots of the supernatant were evaporated and cyclic AMP was measured by a kit cyclic AMP assay system.

**Calculations and statistics**

Binding data were analyzed by the method of Scatchard [22] using the nonlinear curve-fitting program LIGAND [23]. The results are presented as mean ± S.E.M.
Results

Effect of time, temperature, cell concentration and pH on $^{125}$I-VIP binding and degradation

The specific binding of $^{125}$I-VIP to rat peritoneal macrophages was a time and temperature dependent process (Fig. 1, top). At 30 °C, specific binding of tracer to cells was rapid (half-maximal binding occurred within 5 min), became maximal at 30 min and then declined slowly. Reducing the incubation temperature from 30 to 15 °C produced a decrease in the rate of the binding reaction (half-maximal binding was attained after 30 min) but the maximum amount of tracer bound was very similar at both temperatures. The apparent equilibrium state was attained at about 90 min and was maintained until 120 min. Degradation of $^{125}$I-VIP was also a time and temperature-dependent process (Fig. 1, bottom). The degradation of tracer at 30 °C was increased compared to that observed at 15 °C, explaining at least in part the decrease of binding observed at 30 °C. In subsequent experiments, incubation of cells was performed at 15 °C for 90 min to provide a maximal binding and a minimal inactivation of the tracer.

The specific binding of $^{125}$I-VIP was a linear function ($r = 0.98$) of cell concentration up to $2 \cdot 10^6$ cells/ml (Fig. 2, top). At higher cell concentrations the observed binding was less than expected, probably because the degradation of tracer was relatively high (Fig. 2, bottom). Further incubations were then performed with $1.5 \cdot 10^6$ cells/ml, i.e., when only about 15% of tracer was degraded. Preincubation of cells in the absence of peptide resulted in a progressive decrease of the binding activity, probably due to inactivation of VIP receptors. Cells that had been incubated with buffer alone for

Fig. 3. Time-course of dissociation of $^{125}$I-VIP from rat peritoneal macrophages. Cells ($1.5 \cdot 10^6$ cells/ml) were incubated with 45 pM $^{125}$I-VIP at 15 °C for 90 min prior to dissociation. Then, 1 μM native VIP was added and the radioactivity specifically bound to the cells was determined at appropriate times and expressed as the percentage of that at time zero. Each point is the mean of triplicates. Two other separate experiments gave similar results.
90 min, retained 80% of their ability to bind peptide (data not shown). Binding reaction also depended on the pH of the incubation medium. Binding occurred over a relatively large range of pH and was maximal at pH 7.5 (data not shown).

In order to examine the reversibility of the binding reaction, dissociation of the tracer-cell complex was studied by the addition of 1 μM VIP to the incubation mixture at steady state (Fig. 3). The time-course of dissociation did not follow simple first-order kinetics. The dissociation pattern can be explained by the existence of more than one class of VIP receptors; the dissociation of ^125^I-VIP can be considered as the sum of two first-order processes with apparent rate constants of about $3.3 \cdot 10^{-3}$ and $2.3 \cdot 10^{-2}$ min$^{-1}$ for the high affinity-slow dissociating and the low affinity-fast dissociating site, respectively.

**Competitive inhibition of ^125^I-VIP binding by unlabelled VIP and other peptides**

When increasing concentrations of unlabelled VIP were added simultaneously with a fixed concentration (45 pM) of ^125^I-VIP to the incubation medium, a competitive binding to rat peritoneal macrophages was observed (Fig. 4). Half-maximal inhibition (IC$_{50}$) was obtained at about $1.9 \pm 0.6$ nM native VIP. The Scatchard [22] plot of the steady-state binding data was curvilinear with upward concavity (Fig. 4, inset). This course could be resolved into two straight lines suggesting the presence of two different classes of VIP binding sites: a class with high affinity ($k_d = 1.1 \pm 0.1$ nM) and low

![Figure 4](image)

**Fig. 4.** Competitive displacement of ^125^I-VIP binding to rat peritoneal macrophages by unlabelled VIP. Cells (1.5 \cdot 10^6 cells/ml) were incubated with 45 pM ^125^I-VIP at 15 °C for 90 min in the absence or presence of increasing concentrations of unlabelled VIP. Results are the means ± S.E.M. of 12 separate experiments. In each of the experiments, determinations were made in triplicate. A Scatchard [22] analysis of the data is shown in the inset.
Specificity of VIP receptors in rat peritoneal macrophages was investigated by determining the specific binding of $^{125}$I-VIP in the presence of peptides structurally related or unrelated to VIP (Fig. 5). Both rat and human growth hormone releasing factors (rGRF and hGRF, respectively) were able to inhibit tracer binding but with about a 20- and 200-fold lower potency than rat VIP. Peptide histidine isoleucine amide (PHI) was also able to inhibit tracer binding but with a very low potency. Secretin, another peptide structurally related to VIP, induced a slight displacement of $^{125}$I-VIP at a concentration as high as 1 μM. On the other hand, neither glucagon, which is structurally related to VIP nor other structurally unrelated peptides such as somatostatin, insulin, octapeptide of cholecystokinin and pancreastatin, showed any effect at concentrations up to 1 μM.

**Effect of VIP on cyclic AMP production**

VIP activated cyclic AMP production by rat peritoneal macrophages in a dose-dependent and monophasic manner (Fig. 6). The response occurred in the 0.01–1000 nM range of VIP concentrations. Half-maximal stimulation (ED$_{50}$) was elicited at about 1.2 ± 0.5 nM and maximal stimulation (about 3-fold basal levels) was obtained between 0.1–1 μM. As shown in the inset of Fig. 6, Hill plot [24] of the dose-response data was a straight line with a Hill coefficient of 0.99.

![Fig. 5. Competitive inhibition of $^{125}$I-VIP binding to rat peritoneal macrophages by unlabelled VIP (●), rat growth hormone releasing factor (rGRF) (○), human growth hormone releasing factor (hGRF) (■), peptide histidine isoleucine amide (PHI) (□), secretin (▲), glucagon (△), insulin (Θ), somatostatin (◆), pancreastatin (⋆) and octapeptide of cholecystokinin (CCK 26–33) (☆). Binding is expressed as the percentage of radioactivity specifically bound in the absence of unlabelled peptide. Each point is the mean of five separate experiments performed in triplicate. For clarity, standard errors are not indicated; they are always below 10% of the mean values.](image-url)
Fig. 6. Effect of increasing concentrations of VIP on cyclic AMP production in rat peritoneal macrophages. Cells (1·10^6 cells/ml) were incubated in the presence of 0.02 mM IBMX and increasing concentrations of VIP for 45 min at 15 °C. Results are the means ± S.E.M. of six separate experiments. In each of the experiments, determinations were made in triplicate. The inset shows a Hill analysis [24] of the same data. S is the cyclic AMP production above basal for each concentration of VIP and S_M is that for the maximally active concentration of peptide.

Discussion

This study provides evidence for the first time, by means of a number of well established criteria, the existence of functional VIP receptors in rat peritoneal macrophages. Peritoneal macrophages were elicited from Wistar rats according to the Tsunawsky and Nathan method [18]. This method has been well documented by various authors for isolation of peritoneal macrophages [25,26]. Kinetic and stoichiometric studies showed that the binding reaction depended on time, temperature and pH, and was saturable, reversible and specific, in well agreement with that observed in human blood mononuclear cells and monocytes [9–12], mouse lymphocytes [13] and rat lymphoid cells [14]. Binding studies were performed under optimal experimental conditions since receptor degradation was only of 20% at 90 min and 15 °C, whereas peptide degradation did not exceed 10% under similar conditions. The relatively high nonspecific binding is presumably due to internalization and nondisplaceability of the intracellular 125I-VIP, such as has been demonstrated in HT29 cells [27]. However, this process has not been investigated in the present work.

As suggested by Scatchard analysis [22] of the binding data and dissociation experiments, two independent classes of VIP receptors can be defined: a class with high-affinity (k_d = 1.1 ± 0.1 nM) and low binding capacity (11.1 ± 1.5 fmol/10^6 cells) that
represents about 3% of total binding capacity, and a class with low-affinity 
\( k_d = 71.6 \pm 10.2 \text{ nM} \) and high binding capacity \( (419.0 \pm 80.0 \text{ fmol/10}^6 \text{ cells}) \). This 
result is similar to those obtained in human blood mononuclear cells and monocytes 
[9,11,12], rat lymphoid cells [14] and rabbit spleen lymphocytes [15]. However, this 
is in contrast to results obtained from Danek [10], Ottaway [13] and Beed [28], who 
observed a single class of high-affinity VIP receptor in human nonadherent lympho-
cytes, mouse lymphocytes and Molt 4b T-lymphoblasts, respectively. This difference 
may be due to different ionic composition of the incubation buffer, different incubation 
temperature or different methods of calculating \( k_d \) and binding capacity. However, this 
disagreement does not appear to be significant since only the coupling of VIP receptors 
with high-affinity are functionally coupled to the stimulation of cyclic AMP production 
[9,11] and activation of cyclic AMP-dependent protein kinase [17]. With respect to 
specificity, several peptides structurally and not structurally related to VIP were investi-
gated by determining the inhibition of \(^{125}\text{I-VIP} \) specific binding. The following order of 
potency was observed: VIP > rGRF > hGRF > PHI > secretin. Curiously, hGRF is 
in this system more potent that PHI and secretin. However, similar results have been 
observed by other authors [29–31].

The functional nature of the VIP receptors on rat peritoneal macrophages was 
demonstrated by the ability of VIP in to stimulate cyclic AMP production. Half-
maximal stimulation (ED\(_{50}\)) was elicited about 1.2 \( \pm \) 0.5 nM VIP, a value that is in good 
agreement with the \( k_d \) value (1.1 nM) of the high-affinity VIP receptors. Then, it may 
be that the action of VIP on cyclic AMP production results from the interaction of the 
peptide with the high-affinity receptors, whereas the low-affinity receptors are essentially 
spare and not coupled to adenylate cyclase. A similar feature has been shown for VIP 
in human blood mononuclear cells [9,11]. Furthermore, this argument is supported by 
the fact that Hill plot [24] calculated in cyclic AMP experiments was a straight line with 
a Hill coefficient of 0.99. The demonstration of functional VIP receptors in rat peritoneal 
macrophages suggests that VIP exerts a direct action at this level, but does not offer 
an explanation for their physiological significance. However, present results correlate 
with the fact that macrophages derive from blood monocytes [32] and that VIP recep-
tors have been shown in human monocytes [12]. Further studies are necessary to 
determinate if rat monocytes also possess VIP receptors. Macrophages are able to 
express a remarkable array of plasma membrane receptors which play an important role 
in regulation of several macrophage functions such as growth, differentiation, activation 
and migration [32,33]. Thus, it is interesting to speculate that VIP plays an important 
role in regulation of macrophage function. In this context, it must be kept in mind that 
VIP inhibits the respiratory burst in human monocytes [34,35].

In conclusion, our studies show for the first time that rat peritoneal macrophages 
possess functional VIP receptors. The physiological significance of these results remains 
to be investigated. However, this study further supports an important relationship 
between neural and immune systems.
Acknowledgements

This research was supported in part by a Grant of Consejería de Educación de la Junta de Andalucía (Set.89). We thank Dra. Ana Fernandez for her histological technical assistance.

References