Expression of vasoactive intestinal peptide binding sites in rat peritoneal macrophages is stimulated by inflammatory stimulus

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Received 1 November 1994; revised 23 August 1995; accepted 6 September 1995

Abstract

Vasoactive intestinal peptide (VIP) binding to resident and stimulated-rat peritoneal macrophages was studied. No specific VIP binding was obtained with resident rat peritoneal macrophages. In contrast, VIP bound specifically to casein-elicited macrophages. The Scatchard analysis of binding data was consistent with the presence of two classes of VIP binding sites, but may represent a receptor site and internalized VIP. Both specific VIP binding and number of specific high affinity binding sites for VIP augmented progressively after sodium caseinate injection, reaching maximum at days 4–5. Macrophages obtained 1 day after injection showed a minimal specific VIP binding (0.3 ± 0.1% of total), but cells obtained 4 days after injection showed a maximal binding to the peptide (3.1 ± 0.2% of total). The number of high affinity binding sites per cell raised also progressively after sodium caseinate injection: 2650 ± 301 at day 2, 4939 ± 723 at day 3, 6684 ± 903 at day 4 and 9636 ± 1626 at day 5 (P = 0.0035). The number of low affinity binding sites per cell exhibited the same changes. In contrast, the Kd values of both high and low affinity VIP binding sites did not vary significantly (P > 0.05). These results demonstrate that VIP binding sites are only displayed by stimulated macrophages, suggesting that VIP binding sites could be considered to be a pre-activation marker in macrophages and could be used to recognize inflammatory or stimulated macrophages.

Keywords: Vasoactive intestinal peptide (VIP); Rat peritoneal macrophages; Stimulated macrophages; VIP binding site; Neuroimmunoregulation; Cell communication

1. Introduction

Recently we have described specific vasoactive intestinal peptide (VIP) binding sites coupled to adenylate cyclase in rat and mouse peritoneal macrophages (Segura et al., 1991, 1992a; Calvo et al., 1994a,b). The cells preparation used in these studies were obtained by the method of Tsunawaki and Nathan (1984), washing the peritoneal cavity of animals with 0.9% NaCl 4 days after intraperitoneal injection of 5 ml of 6% sodium caseinate. Macrophages obtained by this procedure are called 'casein-elicited macrophages' (Cohn, 1978) and are considered 'non-specifically activated macrophages', 'stimulated macrophages', 'inflammatory macrophages' or 'primed macrophages' (Karnovsky and Lazdins, 1978; Tsunawaki and Nathan, 1984; Hamilton and Adams, 1987). On the other hand, macrophages obtained by washing the peritoneal cavity of unmanipulated animals are called 'resident peritoneal macrophages' or 'unstimulated peritoneal macrophages' (Cohn, 1978). Stimulated macrophages represent a pre-activation state. They have a larger size than resident macrophages, which is expressed in its increased protein content, and showed markedly altered exteriorly disposed plasma membrane polypeptides (Cohn, 1978), showing as markers the presence of the lymphocyte function-associated antigen-1 (LFA-1), the class II histocompatibility antigens I-A, and the absence of the transferrin receptor (Hamilton and Adams, 1987).

The regulation of expression and number of receptor molecules at the cell surface is a common way to modulate cell responsiveness to an external signal. In this context, it has been demonstrated that binding sites for VIP on human mononuclear leukocytes are upregulated during prolonged strain and energy deficiency (Wiik, 1988) and that glucocorticoids increased the number of VIP binding sites in mononuclear leukocytes without changing its Kd value (Wiik, 1991). Moreover, in T and B lymphocytes, the...
membrane-bound receptor specific for insulin, is only expressed after specific antigenic stimulation, when lymphocytes become activated (Helderman and Strom, 1978; Braciale et al., 1982).

The purpose of this paper is to study the presence of VIP binding sites in resident and stimulated rat peritoneal macrophages in order to determine if the inflammatory stimulus represented by sodium-caseinate injection regulates the expression of VIP binding sites in these inflammatory cells.

2. Materials and methods

2.1. Chemicals

Synthetic rat VIP was purchased from Peninsula Laboratories Europe (Merseyside, UK); bacitracin and bovine serum albumin (fraction V) were purchased from Sigma Chemical (St. Louis, MO). Carrier-free Na $^{125}$I (IMS 30, 100 mCi/ml) was obtained from Radiochemical Center (Amersham, UK). Synthetic rat VIP was radioiodinated by the chloramine T method as previously described (Segura et al., 1991) with a specific activity of 832 Ci/mmol. Purification of labeled tracer was performed on a Sephadex G-50 column (1 × 30 cm) eluted with 0.2 M acetic acid containing 0.2 M acetic acid containing 0.5% (w/v) BSA and 0.03% (w/v) bacitracin.

2.2. Collection of rat peritoneal macrophages

Peritoneal macrophages were elicited from Wistar rats according to the method of Tsunawaki and Nathan (1984). Utmost precautions were taken such that the animals remained free from infection by environmental pathogens. Briefly, female Wistar rats, unmanipulated or injected 1, 2, 3, 4, 5, 6 or 7 days before harvest with 5 ml of 6% sodium caseinate, were killed by decapitation and, immediately, the peritoneal cavity was washed with 10 ml of cold 0.9% NaCl, the cell exudate was removed with a syringe and centrifuged for 10 min at 250 × g and 4°C. The contaminating red blood cells were lysed with cold 0.2% NaCl. Cells were then washed with 0.9% NaCl by centrifugation and counted. By morphological criteria in Giemsa and Papanicolaou staining techniques (Wintrobe, 1974) cells were identified as macrophages (▲), lymphocytes (●), or polymorphonuclear leukocytes (■).

2.3. Binding studies

Binding experiments were carried out according to the method described previously (Segura et al., 1991). Briefly, under standard conditions, peritoneal macrophages (1.5 × 10$^6$ 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 $^{125}$VIP either alone (total binding) or together with 10$^{-6}$ M unlabelled VIP (nonspecific binding). After 90 min incubation, cell-bound peptide was separated by centrifugation, as described previously (Calvo et al., 1986b), 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.

2.4. Calculations and statistics

Statistical significance was determined by one-way analysis of variance (ANOVA test). Binding data were analyzed by the method of Scatchard (1949) using the nonlinear curve-fitting program LIGAND (Munson and Rodbard, 1980).

3. Results

3.1. Cellular composition of peritoneal exudates

Peritoneal exudates obtained from unmanipulated and caseinate-injected animals were studied in order to determine their cellular composition. By morphological criteria in Giemsa and Papanicolaou staining techniques (Wintrobe, 1974) cells were identified as macrophages (▲), lymphocytes (●), or polymorphonuclear leukocytes (■).
3.2. VIP binding to resident and casein-elicited rat peritoneal macrophages

When resident macrophages (1.5 × 10^6 macrophages/ml) were incubated with 45 pM [125I]VIP alone, no specific binding was obtained (Fig. 2). Macrophages obtained 1 day after caseinate injection showed a minimal binding capacity to tracer (specific binding = 0.3 ± 0.1%). On the contrary, [125I]VIP bound specifically to macrophages increased clearly since the 2nd day. Both total and specific binding increased progressively up to the 4th day after injection of sodium caseinate. After 2, 3, 4, 5, 6 and 7 days, specific VIP binding to rat peritoneal macrophages was 0.8 ± 0.2%, 1.4 ± 0.1%, 3.1 ± 0.2%, 2.5 ± 0.3%, 1.8 ± 0.1%, and 1.4 ± 0.1%, respectively. Nonspecific VIP binding varied from 1.0% at day 1 to 1.8% at day 4.

3.3. Correlation between specific VIP binding and percentage of each cell type

In order to verify that macrophages bound [125I]VIP specific VIP binding to peritoneal exudate cells was correlated with proportion of each cell type (Fig. 3). Specific VIP binding was found to be a direct function of macrophage percentage (r = 0.98). In contrast, no correlation was observed between specific VIP binding and lymphocytes (r = 0.13) or polymorphonuclear leukocytes (r = 0.37).

3.4. Stoichiometric studies

In the stoichiometric studies, casein-elicited rat peritoneal macrophages were incubated with tracer in the presence of increasing concentrations of unlabelled VIP (Fig. 4). The VIP concentrations that inhibited [125I]VIP binding to macrophages by 50% (IC50) were very similar and of the same range in all cases: 0.79 ± 0.1 nM at day 2, 0.62 ± 0.07 nM at day 3, 1.01 ± 0.2 nM at day 4, 1.13 ± 0.2 nM at day 5, 1.29 ± 0.5 nM at day 6 and 0.73 ± 0.08 nM at day 7.

The Scatchard analysis of the stoichiometric data gave a curvilinear plot in all cases (Fig. 5) that could be resolved into two straight lines, suggesting the presence of two different classes of VIP binding sites: a class with high affinity and low binding capacity and another class with low affinity and high binding capacity. However, either may represent a receptor site and internalized VIP.
Fig. 5. Scatchard analysis of the specific binding of [125I]VIP to peritoneal macrophages. Scatchard plot of the stoichiometric data of binding experiments represented in Fig. 4 are shown. Nonspecific binding was measured as the binding that could be displaced by 1 μM VIP. The curvilinear plots can be resolved into two linear components from which $K_d$ and $B_{max}$ may be calculated.

apparent equilibrium dissociation constants ($K_d$) and binding capacities ($B_{max}$) were calculated from the stoichiometric data using the nonlinear curve-fitting program EBDA-LIGAND (Munson and Rodbard, 1980) and are shown in Table 1. As it can be seen, the $K_d$ values obtained for the high affinity binding sites were of the same range and comparable in all cases. Applying the ANOVA test, differences among the group means were not significant ($P = 0.3862$). In contrast, $B_{max}$ and the number of binding sites per cell increased clearly from the 2nd (2650 ± 301) to the 5th day (9636 ± 1626), and decreased slowly at day 6 (7950 ± 1265) and day 7 (6866 ± 964). Applying the ANOVA test, highly significant differences among the group means were obtained ($P = 0.0035$).

The $K_d$ values calculated for the low affinity binding sites were also analogous in all cases, without significant differences ($P = 0.2275$) and the binding capacities obtained showed the same variation observed in the high affinity binding sites, with very significant differences among the group means ($P = 0.0046$).

### Table 1

<table>
<thead>
<tr>
<th>Day</th>
<th>High affinity binding sites</th>
<th>Low affinity binding sites</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_d$ (nM)</td>
<td>$B_{max}$ (fmol/10⁶ cells)</td>
</tr>
<tr>
<td>2</td>
<td>0.88 ± 0.1</td>
<td>4.4 ± 0.5</td>
</tr>
<tr>
<td>3</td>
<td>1.02 ± 0.1</td>
<td>8.2 ± 1.2</td>
</tr>
<tr>
<td>4</td>
<td>1.10 ± 0.2</td>
<td>11.1 ± 1.5</td>
</tr>
<tr>
<td>5</td>
<td>0.98 ± 0.1</td>
<td>16.0 ± 2.7</td>
</tr>
<tr>
<td>6</td>
<td>1.19 ± 0.3</td>
<td>13.2 ± 2.1</td>
</tr>
<tr>
<td>7</td>
<td>1.43 ± 0.4</td>
<td>11.4 ± 1.6</td>
</tr>
</tbody>
</table>

Data of Scatchard analysis of binding experiments represented in Fig. 5 were analyzed using the nonlinear curve-fitting program LIGAND (Munson and Rodbard, 1980). The apparent equilibrium dissociation constants ($K_d$) and binding capacities ($B_{max}$) were calculated.

4. Discussion

This paper shows for the first time that VIP binding capacity of macrophages depends upon its stimulation or pre-activation by an inflammatory stimulus, such as sodium caseinate injection. In contrast, unstimulated macrophages does not show specific VIP binding.

Although cellular composition of peritoneal exudates was dependent on days passed after caseinate injection, in all cases cells were identified and adjusted to 1.5 X 10⁶ macrophages/ml. At day 4 after the inflammatory stimulus, the percentage of macrophages was maximum (91 ± 5%). This is in accordance with previous reports that used peritoneal exudate cells to characterize the VIP receptor-effector system in rat and mouse peritoneal macrophages (Segura et al., 1991, 1992a; Calvo et al., 1994b). One and two days after caseinate injection, the percentage of polymorphonuclear leukocytes was greater than macrophages. However, polymorphonuclear leukocytes neither bind [125I]VIP with high affinity nor demonstrate VIP-mediated stimulation of adenylate cyclase (O'Dorisio et al., 1981; Wiik et al., 1985; O'Dorisio, 1987). Moreover, no correlation was observed between specific VIP binding and percentage of polymorphonuclear leukocytes ($r = 0.37$) and lymphocytes ($r = 0.13$). The percentage of lymphocytes was the lowest in all cases. Thus, although they have been shown to have specific VIP binding sites in rats (Calvo et al., 1986b), mice (Ottaway and Greenberg, 1984) and humans (Ottaway et al., 1990), the proportion of total specific VIP binding to exudate cells corresponding to lymphocytes can be considered minimal. In contrast, specific VIP binding to peritoneal exudate cells only was found to be a direct function of macrophage percentage ($r = 0.98$). Thus, we can accept that cells binding [125I]VIP are macrophages.

No specific VIP binding was found in resident macrophages. However, VIP binding to stimulated-macrophages was dependent on time elapsed from sodium-caseinate injection to collection of peritoneal macrophages. Total and specific VIP binding was maximum at day 4. The relatively high nonspecific binding was probably due...
to internalization and nondisplaceability of the intracellular [125I]VIP, such as has been demonstrated in HT29 cells (Boissard et al., 1984) and in human leukocytes (Wiik, 1988). Other studies on receptor characterization carried out on macrophages, also have found high values of nonspecific binding (Abrass et al., 1985; Segura et al., 1991; Calvo et al., 1994b).

As suggested by Scatchard analysis of the binding data, two independent classes of VIP binding sites can be defined in all cases for stimulated macrophages: a class with high affinity and low binding capacity and a class with low affinity and high binding capacity. These results are similar to those obtained in human blood mononuclear cells and monocytes (Guerrero et al., 1981; Wiik et al., 1985), rat lymphoid cells (Calvo et al., 1986b), rabbit spleen lymphocytes (Peuriere et al., 1990), and rat and mouse peritoneal macrophages (Segura et al., 1991; Calvo et al., 1994b). However, this is in contrast to results obtained by Ottaway and Greenberg (1984), Beed et al. (1983) and Blum et al. (1992), who observed a single class defined in all cases for stimulated macrophages: a class with high affinity and low binding capacity and a class with low affinity and high binding capacity. The increase in the number of high and low affinity VIP binding sites on macrophages was observed after the inflammatory stimulus caused by intraperitoneally injection of 6% sodium caseinate. The increase was time-dependent, reaching a maximum at day 5 and 4 for the high and low affinity binding site, respectively. No significant changes in $K_d$ values were observed, being of the same range of these calculated previously for monocytes (Wiik et al., 1985), rat peritoneal macrophages (Segura et al., 1991, 1992b; Calvo et al., 1994a) and mouse peritoneal macrophages (Calvo et al., 1994b). The absence of significant changes in the $K_d$ values of both high and low affinity binding sites suggests that the inflammatory stimulus originated by casein injection did not change the molecular characteristics of binding sites, but only increased their number.

Cell preparations used in this study were obtained by washing the peritoneal cavity of rats with 0.9% NaCl at different days after intraperitoneal injection with 5 ml of 6% sodium caseinate. The cell suspension employed in the experiments contained the same number of macrophages since cells were adjusted in 0.9% NaCl at 1.5 x 10^6 macrophages/ml. Macrophages obtained by this procedure are considered 'nonspecifically activated macrophages', 'stimulated macrophages', 'inflammatory macrophages' or 'primed macrophages' and represent a pre-activation state (Karnovsky and Lazdins, 1978; Hamilton and Adams, 1987).

Most of the studies carried out on macrophages, including our own works, have been performed using casein or thioglycolate-elicited macrophages (stimulated macrophages) in order to obtain a higher number of cells in the peritoneal cavity exudate (De La Fuente et al., 1993a; Segura et al., 1993; Calvo et al., 1994b). Results demonstrate that conclusions of receptor system studies obtained in stimulated macrophages cannot be assumed directly for unstimulated macrophages because one of the most important functional changes occurred during pre-activation is a marked alteration of the exteriorly disposed plasma membrane polypeptides (Cohn, 1978). During this process the lymphocyte function-associated antigen-1 (LFA-1) and the class II histocompatibility antigens I-A are expressed (Hamilton and Adams, 1987). The contradictory results observed in studies on macrophage function performed with peritoneal or alveolar macrophages (Litwin et al., 1992; De La Fuente et al., 1993b; Segura et al., 1993) could be explained by the fact that alveolar macrophages are not stimulated, whereas casein-elicited peritoneal macrophages are stimulated cells. At this respect, studies on VIP stimulation of adenylate cyclase activity in normal analysis of binding data may not represent two different receptor types of differing affinity, but rather may represent a receptor site and internalized VIP.

Using the nonlinear curve-fitting program LIGAND (Munson and Rodbard, 1980), the $K_d$ values and the numbers of these two classes of VIP binding sites were calculated. An increment in the number of high and low affinity VIP binding sites on macrophages was observed after the inflammatory stimulus caused by intraperitoneally injection of 6% sodium caseinate. The increase was time-dependent, reaching a maximum at day 5 and 4 for the high and low affinity binding site, respectively. No significant changes in $K_d$ values were observed, being of the same range of these calculated previously for monocytes (Wiik et al., 1985), rat peritoneal macrophages (Segura et al., 1991, 1992b; Calvo et al., 1994a) and mouse peritoneal macrophages (Calvo et al., 1994b). The absence of significant changes in the $K_d$ values of both high and low affinity binding sites suggests that the inflammatory stimulus originated by casein injection did not change the molecular characteristics of binding sites, but only increased their number.

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and leukemic human monocytes and macrophages showed that VIP did not affect cyclic AMP generation in monocyte-derived macrophages or in alveolar macrophages (Chedeville et al., 1993). In contrast, VIP has been shown to increase cyclic AMP production in rat and mouse inflammatory macrophages (Segura et al., 1992a,c; Calvo et al., 1994b) and to stimulate adenylate cyclase activity in human peripheral monocytes (Chedeville et al., 1993).

Helderman and Strom (1978) showed that insulin binding sites in lymphocytes are only expressed by activated cells. This finding conducted the authors to propose insulin binding sites as a marker of lymphocyte activation. Similarly, our results demonstrate that unstimulated or resident macrophages do not possess specific VIP binding sites on the cell surface but, when they become pre-activated by an inflammatory stimulus, such as caseinate injection, the expression of specific VIP binding sites occurs and they develop the capacity of binding VIP. Thus, the expression of specific VIP binding sites on macrophage could be also considered as a marker of its pre-activation state.

It is well known that VIP is released in mast cells by histamine liberators (Cutz et al., 1978). Moreover, the presence of immunoreactive VIP in polymorphonuclear leukocytes and lymphoid cells has been shown (O’Dorisio et al., 1980; Leceta et al., 1994) and, recently, it has been shown the VIP mRNA expression in rat T and B lymphocytes (Gomariz et al., 1994), suggesting that immune cells may be an important source of VIP in regional lymphatic tissues. These findings, together with our present and previous results (Guerrero et al., 1981; Calvo et al., 1986b, 1994b; Segura et al., 1991, 1992a,c, 1993), suggest that expression of VIP binding sites in macrophages is stimulated by inflammatory stimulators, supporting the concept that VIP is involved in communication between immunocompetent and inflammatory cells, and suggesting that during inflammation and immune response VIP acts as a cytokine, being secreted by lymphocytes and/or mast cells and, acting in a paracrine mode, binding to macrophages, and modulating their capacities.

Acknowledgements

Supported by grants from Dirección General de Investigación Científica y Técnica (PB94-1434) and Fondo de Investigaciones Sanitarias de la Seguridad Social (FISSSS 93/0210).

References


approach for characterization of ligand-binding systems. Anal.
Biochem. 107, 220–239.

O’Dorisio, M.S. (1987) Biochemical characteristics of receptors for va-
soactive intestinal polypeptide in nervous, endocrine and immune

VIP as a biochemical marker of polymorphonuclear leukocytes. J.

O’Dorisio, M.S., Hermina, N.S., O’Dorisio T.M. and Balcerzak, P.
(1981) Vasoactive intestinal polypeptide modulation of lymphocyte

Ottaway, C.A. (1992) Receptors for vasoactive intestinal peptide on

intestinal peptide with mouse lymphocytes: specific binding and the

Specific binding of vasoactive intestinal peptide to human circulating

specific binding of vasoactive intestinal peptide to human circulating
T cells, B cells and large granular lymphocytes. J. Neuroimmunol. 29,
149–155.

Peuriere, S., Susini, Ch., Esteve, J.-P., Vaysse, N. and Escoula, L. (1990)
Dual effect of vasoactive intestinal peptide on the mitogenic response

of vasoactive intestinal peptide (VIP) receptors on rat peritoneal

Scatchard, G. (1949) The attractions of proteins for small molecules and

terization of functional receptors for vasoactive intestinal peptide

tory effect of vasoactive intestinal peptide (VIP) on cyclic AMP

nine nucleotide regulation of VIP binding to rat peritoneal macrophage

Segura, J.J., Guerrero, J.M., Gobena, R. and Calvo, J.R. (1992c) So-
motostatin inhibition of VIP- and isoproterenol-stimulated cyclic AMP
production in rat peritoneal macrophages. Neuropeptides 73. 99–43

(1993) Vasoactive intestinal peptide (VIP) inhibits substrate adherence
capacity of rat peritoneal macrophages by a mechanism that
involves cAMP. Cell Adh. Commun. 1, 213–221.


receivers for vasoactive intestinal peptide (VIP) in human mononu-

Wiik, P. (1991) Glucocorticoids upregulate the high affinity receptors for
vasoactive intestinal peptide (VIP) on human mononuclear leucocytes

intestinal polypeptide (VIP) by human blood monocytes: demon-