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Original Article

Non-specific activation of human eosinophil functional responses by vasoactive intestinal peptide

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Abstract

Eosinophils and neuropeptides are thought to play effector roles in allergic diseases, such as rhinitis; however, little is known about the biological effects of neuromediators, especially vasoactive intestinal peptide (VIP), on eosinophil functional responses. In the present study, it is shown that VIP induces eosinophil chemotaxis and eosinophil-derived neurotoxin (EDN) release in potency comparable with that induced by platelet activator factor, and in a novel synergistic manner with recombinant human interleukin-5. Contrary to chemotaxis, EDN release was sensitive to staurosporine, the protein kinase C inhibitor, as well as intracellular calcium chelation. However, eosinophil treatment with inhibitors of tyrosine kinases (herbimycin A) and phosphatases (pervanadate) resulted in a dose-dependent potentiation and blockage of VIP-induced eosinophil chemotaxis, respectively. Treatment of eosinophils with VIP receptor antagonist did not modify VIP-induced chemotaxis or EDN release. Furthermore, exploration of vasoactive intestinal peptide receptor I expression was lacking in human eosinophils, but not lymphocytes. These results demonstrate two different mechanisms in triggering eosinophil activation of functional responses by VIP, a calcium-dependent degranulation and a calcium-independent chemotaxis, and elaborate on a novel cytokine–neuropeptide interaction in eosinophilic inflammation.

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Introduction

Eosinophils contain four predominant cationic molecules, including the eosinophil peroxidase (EPO), the major basic protein (MBP), the eosinophil cationic protein (ECP), and the eosinophil-derived neurotoxin (EDN). Eosinophil accumulation and the release of their granule protein contents in response to the proper mediators is a crucial factor in the pathophysiology of allergic inflammation.[1–3]

In the past decade or so, intensive research in the fields of neuropeptides and immune cells has resulted in accumulating evidence that supports the existence of a neuroimmune axis.[4–6] However, the biological effects of vasoactive intestinal peptide (VIP) on human eosinophil are not fully characterized and it is not clear whether VIP possesses an anti-inflammatory or deleterious effect in hypersensitivity reactions.[7,8]

The 28 amino acid VIP polypeptide, which exists in the parasympathetic nerves and to a lesser extent in the sensory fibers, is one of the most abundant of the neuropeptides found in the upper and lower airways[9,10] and has been detected in the fluids of hypersensitivity reactions.[11] The biological effects of VIP on airway functions include regulation of vascular tone, airway caliber and mucus secretion.[12–15] In addition to its direct physiologic effects on target tissues, VIP also has the potential to modulate T lymphocyte functions.[16,17] Although VIP affects T lymphocytes through its specific receptor, its effects on other immune and inflammatory cells is still obscure and there have not been any detailed reports of its effect on eosinophil chemotaxis or exocytosis.

Hemopoietic cytokines, such as IL-5, are known for being specific for human eosinophil differentiation and for prolonging cell survival in culture, and possess a strong priming effect on eosinophils, preparing the cell for an exaggerated inflammatory response to other inflammatory mediators.[18,19] Nonetheless, little is known about cytokine–neuropeptide interactions in eosinophilic inflammation.

In the present study, we investigated the effects of VIP, in the absence or presence of interleukin (IL)-5, on human eosinophil chemotaxis and degranulation. Release of EDN was chosen as a degranulating marker for its relatively easy induction *in vitro*. Results are discussed in relation to the possible molecular pathway(s) involved.

METHODS

Reagents

Percoll solution and EDN radioimmunoassay (RIA) kits were purchased from Pharmacia (Uppsala, Sweden). NP-40 was from Iwai Kagaku Co., Ltd. (Tokyo, Japan). CD16 microbeads were from Miltenyi Biotec (Bergisch Gladbach, Germany). Cytochalasin B and VIP were from Sigma Chemical Co. (St Louis, MO, USA). The VIP receptor antagonist was from Peninsula Laboratories, Inc. Herbimycin A, staurosporine, BAPTA-AM and sodium orthovanadate were from Wako Pure Chemical Industries (Osaka, Japan). Recombinant human (rh)IL-5 was from Genzyme Corporation Chemicals (Cambridge, UK).

Cell preparation

Eosinophils and lymphocytes from healthy volunteers were purified from venous blood anticoagulated with heparin by Percoll discontinuous gradient centrifugation followed by negative selection with CD16 immunomagnetic beads for high purification of eosinophils, as described elsewhere.[20] Briefly, blood was sedimented with 6% dextran and the buffy coat was collected and washed twice with Hank's balanced salt solution (HBSS) containing 2% fetal calf serum (FCS) and cells were overlaid on a discontinuous Percoll gradient of 78%, 71% and 56% Percoll solution in 15 mL polystrene tubes. The tubes were then centrifuged at 400 *g* at 20°C for 30 min, resulting in the formation of three bands and a pellet. The pellet and the lowest band granulocytes were collected and sedimented red blood cells were removed by hypodense lysis. These lymphocyte- and monocyte-free granulocytes were incubated with CD16 immunomagnetic beads for 30 min at 6–12°C. CD16 is expressed on neutrophils, but not on normal resting eosinophils. Cells were applied to the top of a prewashed column MACS separator (Miltenyi Biotec). The CD16 negative cells were collected as 98% pure eosinophilic granulocytes as judged by Hinkelmann staining. Eosinophils showed a viability greater than 97%, which was confirmed by trypan blue dye exclusion. As for lymphocytes, cells were recovered from the upper band.

Chemotaxis assay

Chemotactic experiments were performed in 48-well microchemotaxis chambers as described precisely by us elsewhere.[21] Results of the chemotaxis experiments were expressed as eosinophils/five selected high power fields (5 h.p.f.).

Eosinophil degranulation and assay of EDN concentration

Eosinophil degranulation was induced by VIP or PAF using 96-well flat-bottom culture plates. Freshly purified eosinophils were washed twice in RPMI-1640 containing 10% FCS and were resuspended in the same culture medium at 5×10^5 cells/mL. During preliminary studies, eosinophils adhering to culture plates released large amounts of EDN spontaneously, probably due to non-specific activation, which was inhibited by the addition of 5 μg/mL cytochalasin B to the assay system. Aliquots of cell suspension (100 μL) were added onto the culture plate and degranulation was initiated by adding 100 μL VIP or PAF diluted in the same culture medium as eosinophil and adjusted to the desired concentrations. After 1 h incubation at 37°C and 5% CO_2, supernatants from wells were collected carefully and stored at – 20°C until assayed. Concentrations of EDN in the supernatants were analyzed by RIA using EDN RIA kits (Pharmacia). The total content of EDN was measured by lysing eosinophils with 1% Nonidet P-40.

Priming experiments with rhIL-5

Eosinophils were incubated with 1 ng/mL rhIL-5 for 30 min at 37°C. After this, cells were washed twice and resuspended in the culture medium and their chemotaxis and EDN degranulation by VIP were performed in the same way as described earlier.

Ca^{2+}-depleted eosinophils

Ca^{2+}-depleted eosinophils were obtained by incubating 10^7 cells/mL with 30 μmol/L of the calcium chelating agent BAPTA-AM in test medium (130 mmol/L NaCl, 5 mmol/L $NaHCO_3$, 4.6 mmol/L KCl, 5 mmol/L glucose, 2 mmol/L ethyleneglycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) and 20 mmol/L *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), for 30 min at 37°C.

Total RNA extraction and cDNA synthesis

Total cellular RNA of eosinophils and lymphocytes was extracted using the guanidium isothiocynate method; 1 μL total cellular RNA, 1 μL (500 ng) Oligo(dt) 12–18 primer supplied in diethyl pyrocarbonate (DEPC)-treated water and 3 μL sterile distilled water were heated to 70°C for 10 min followed by quick chilling on ice and were made up to a total volume of 19.3 μL containing first strand buffer, 10 mmol/L deoxyribonucleoside triphosphates (dNTP), 0.1 mol/L dithiothreitol (DTT), and superscript reverse transcriptase (GIBCO BRL). The samples were incubated for 10 min at 37°C and then 50 min at 42°C followed by 5 min at 95°C. Then 0.7 μL (2 U) RNase was added and further incubated for 20 min at 37°C.

Polymerase chain reaction amplification

Aliquots (1 μL) of reverse transcribed mixture were made up to 50 μL with reverse transcriptase (RT) buffer containing 12.5 pmol/μL specific primers and 1 U Taq DNA polymerase. The β-actin and VIP receptor 1 (R1) primers used were exactly similar as those reported by Eura *et al*. and Park *et al*., respectively.[22,23] The samples were overlaid with mineral oil and polymerase chain reaction (PCR) consisted of 1 min of denaturation at 94°C, 2 min of annealing at 60°C and 3 min of extension at 72°C for 40 cycles. The PCR products were analyzed on an ethidium bromide-stained 1.5% agarose gel.

Statistical analysis

Results were expressed as the mean ± SEM. Statistical significance was analyzed by paired Student's *t*-test. $P < 0.05$ was considered statistically significant. All experiments were performed in duplicate.

Results

Vasoactive intestinal peptide-induced eosinophil chemotaxis and EDN release

At a wide range of doses, VIP elicited eosinophil locomotion and EDN release as can be seen in Fig. 1a,b. These effects were comparable with those induced by PAF (Fig. 1c,d). The EDN release showed a time-dependent mode and was not due to cytotoxicity, as judged by trypan blue dye exclusion, which was performed after each degranulation period to check cell viability (data not shown).

A checkerboard assay was performed to identify the nature of eosinophil locomotion in response to VIP. Whenever cells migrate in response to a concentration gradients of chemotactic molecules it is termed chemotaxis, whereas chemokinesis represent random migration without the requirement of a concentration gradient. Figure 2 shows that VIP activity was mainly chemotactic and to a lesser degree chemokinetic at lower concentrations; however, higher concentrations (10^{-5} mol/L and above) demonstrated chemokinetic activity.

Priming effect of rhIL-5

Figure 3 demonstrates the priming effect of 1 ng/mL rhIL-5 on eosinophil chemotaxis and EDN release induced by VIP. Recombinant human IL-5 at the above concentration did not possess any chemotactic or secretagogue activity against eosinophil (data not shown); however, when eosinophils were incubated first with rhIL-5, the cells showed significant sensitivity against VIP to chemotaxis and degranulation rather than chemokinetic activity.

Role of free intracellular calcium as a second messenger in VIP-induced eosinophil chemotaxis and EDN release

Figure 4 illustrates the eosinophilotactic and secretagogue activities of VIP against Ca^{2+}-depleted cells. The quantity of 10^{-6} mol/L VIP was chosen because it is the common dose to induce significantly both functions.

Contrary to EDN release, which was almost completely blocked, eosinophil chemotaxis was not affected under depletion conditions.

Effect of staurosporine on VIP-induced eosinophil chemotaxis and degranulation

Pre-exposure of eosinophils to 100 nmol/L staurosporine prior to VIP induction of eosinophil chemotaxis or

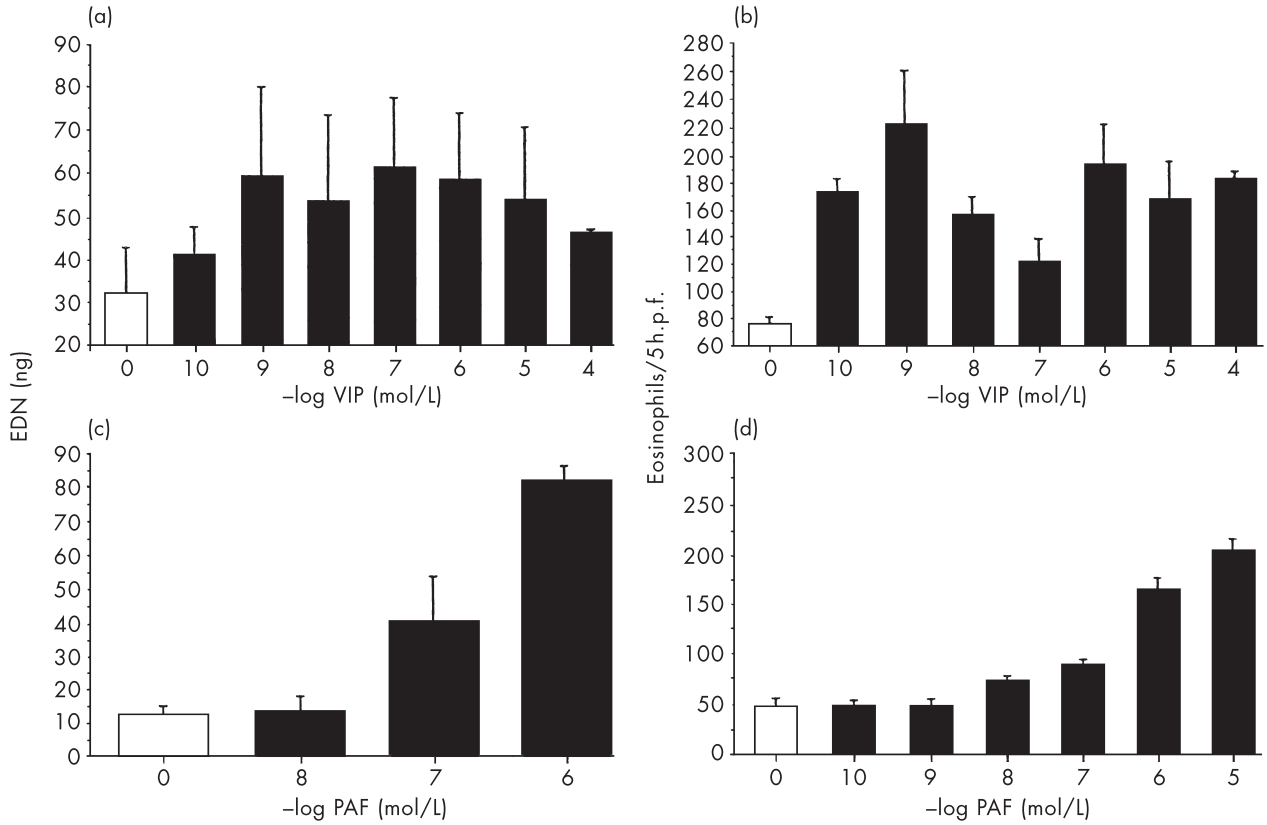

Fig. 1 Comparison of the effects of vasoactive intestinal peptide (VIP) on human eosinophil (a) degranulation and (b) migration. Data are the mean ± SEM, obtained from 5–8 different experiments. $P < 0.05$ for (a) $10^{-4} - 10^{-7}$ mol/L and (b) $10^{-4} - 10^{-10}$ mol/L. Similar dose–response curves were observed in all experiments of chemotaxis. A comparative study on platelet activator factor (PAF) activities is also shown (c,d). EDN, eosinophil-derived neurotoxin; h.p.f., High power field.

Concentration of VIP below filter

Concentration of VIP above filter

-log mol/L	0	10	9	7	6	5	4
0	63	170	223	120	190	165	180
10		100					
9			163				
7				140			
6					160		
5						150	
4							175

Fig. 2 Checkerboard analysis of vasoactive intestinal peptide (VIP) on normal human eosinophils. Values represent the mean number of migrated cells in duplicate filters.

degranulation resulted in a dose-dependent inhibition of EDN release, but did not affect cell migration (Table 1).

Effect of herbimycin A and pervanadate on VIP-induced eosinophil chemotaxis

Recently, several reports assigned a role for tyrosine kinases in leukocyte migration, therefore we next studied their role on VIP-induced eosinophil chemotaxis using herbimycin A, the potent tyrosine kinase inhibitor.[24,25] Cells were incubated with various doses of the inhibitor for 1 h, washed and resuspended in the culture medium and then their chemotactic responses were tested against VIP in the same way as described in Methods. As shown in Fig. 5a, pretreatment of eosinophils with herbimycin A resulted in a dose-dependent potentiation of VIP-induced eosinophilotactic response.

To further gain insight into the role of tyrosine phosphorylation in VIP-induced chemotaxis, eosinophils were

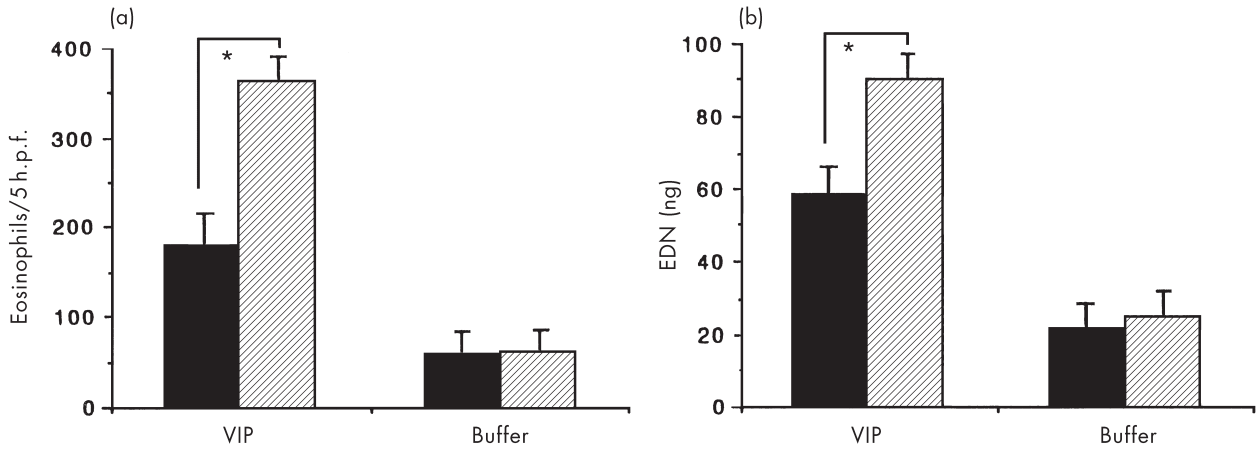

Fig. 3 Priming effect of 1 ng/mL recombinant human interleukin (rhIL)-5 on 10^{-6} mol/L vasoactive intestinal peptide (VIP)-induced eosinophil chemotaxis (a) and degranulation (b). Eosinophils were incubated with the rhIL–5 for 30 min at 37°C, followed by washing of the cells and resuspension in the culture medium. Eosinophil chemotaxis and degranulation against VIP were then tested, n = 6. *$P < 0.01$. Similar results were obtained with 10^{-7} mol/L VIP. (■), No priming; (▨), priming. EDN, eosinophil-derived neurotoxin; h.p.f., high power field.

Table 1 Effect of staurosporine on 10^{-6} mol/L VIP-induced eosinophil chemotaxis and degranulation

Concentration of staurosporine	Eosinophils/5 h.p.f.	EDN (ng)
0 (Buffer)	194.5 ± 14.5	50.0 ± 0.8
100 nmol/L	180.0 ± 8.3	33.0 ± 1.7*

Freshly purified eosinophils were pre-exposed to 100 nmol/L staurosporine for 15 min at 37°C. Results are the mean ± SEM of four different experiments. VIP, vasoactive intestinal peptide; h.p.f., high power field; EDN, eosinophil-derived neurotoxin. *$P < 0.01$.

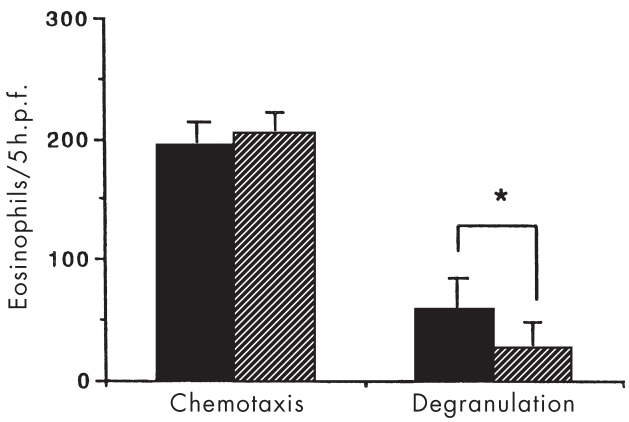

Fig. 4 Comparison of the eosinophilotactic and secretagogue activities of 10^{-6} mol/L vasoactive intestinal peptide (VIP) on Ca^{2+}-depleted eosinophils (▨) versus non-depleted (■). The results represent the mean ± SEM of 9–12 different experiments. *$P < 0.001$. h.p.f., High power field.

treated with pervanadate, the tyrosine phosphatase inhibitor.[26] Pervanadate treatment resulted in a dose-dependent inhibition of VIP-induced chemotaxis, as illustrated in Fig. 5b. As a control, a comparative study on PAF was also performed; however, neither herbimycin A nor pervanadate modulated PAF eosinophilotactic activity (data not shown).

Effect of VIP receptor antagonist on VIP-induced chemotaxis and EDN release

To test whether VIP interaction with human eosinophils is receptor mediated, eosinophils were pretreated with various concentrations of VIP receptor antagonist, [4 Cl-D-Phe^6-Leu^{17}] VIP,[27] for 30 min before induction of chemotaxis and degranulation by VIP. None of the doses tested, from 10^{-5} to 10^{-7} mol/L, modified the activation of the cells by the corresponding agonist (Fig. 6).

Effect of pertussis toxin on VIP-induced eosinophil chemotaxis

Pertussis toxin (PTX) catalyzes ADP-ribophosphorylation of G_i proteins. To examine the role of G_i proteins in VIP-induced eosinophil chemotaxis, eosinophils were pre-incubated with various concentrations of PTX, which *per se* did not affect VIP-induced chemotaxis (Table 2).

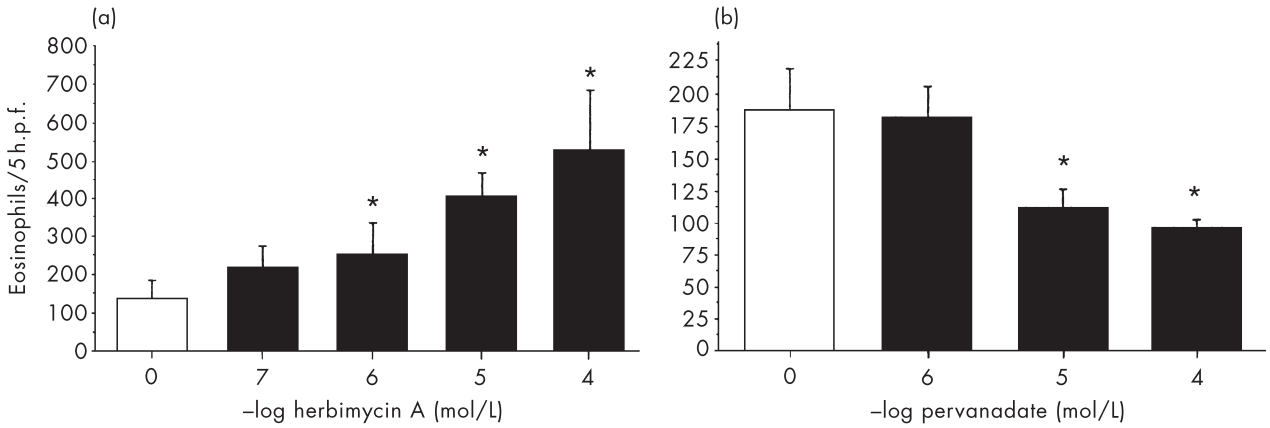

Fig. 5 Effects of herbimycin A (a) and pervanadate (b) on 10^{-6} mol/L vasoactive intestinal peptide (VIP)-induced eosinophil chemotaxis. *$P < 0.05$ compared with control cells that were pre-incubated with buffer for the same duration. Data are the mean ± SEM, $n = 10$. Similar results were obtained with 10^{-9} mol/L VIP. h.p.f., High power field.

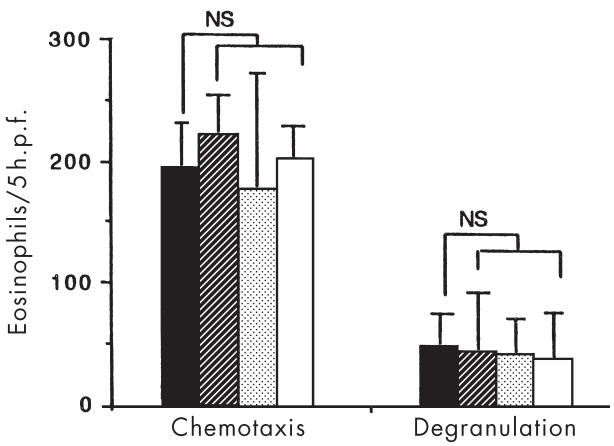

Fig. 6 Effects of vasoactive intestinal peptide (VIP) receptor antagonist on 10^{-6} mol/L VIP-induced eosinophil chemotaxis and degranulation. Cells were pre-incubated with –log 7 (▨), –log 6 (▩) or –log 5 mol/L (□) receptor antagonist or buffer (■; control) for 30 min at 37°C, before adding the corresponding agonist. Results represent the mean ± SEM of six independent experiments. Values did not differ significantly from control cells that were pre-incubated with buffer for the same duration. Similar results were obtained with 10^{-9} mol/L VIP. h.p.f., High power field.

Expression of VIP-R1 by human eosinophils and lymphocytes

The amplification products of VIP-R1 were identical to the predicted size for the mRNA template of 534 b.p. The RT-PCR products from lymphocytes, but not eosinophils, expressed signals for VIP-R1 (Fig. 7).

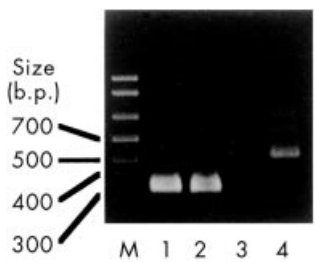

Fig. 7 Expression of *VIP-R1* in human eosinophils and lymphocytes. *VIP-R1* was expressed in lymphocytes but not in eosinophils. Reverse transcriptase polymerase chain reaction (RT-PCR) was performed on mRNA isolated from each cell type. The RT-PCR products were electrophoresed in 1.5% agarose gel and stained with ethidium bromide. M, marker; 1, eosinophils (β-actin); 2, lymphocytes (β-actin); 3, eosinophils (*VIP-R1*); 4, lymphocytes (*VIP-R1*).

Table 2 Effect of PTX on 10^{-6} mol/L VIP-induced eosinophil chemotaxis

Concentration of PTX	Eosinophils/5 h.p.f.
0 (Buffer)	194.5 ± 14.5
10 ng/mL	170.0 ± 5.8
100 ng/mL	182.0 ± 10.2
1000 ng/mL	164.0 ± 16.1

Freshly purified eosinophils were incubated for 2 h at 37°C with various concentrations of pertussis toxin (PTX), followed by washing of the cells and resuspension in the culture medium. Results are the mean ± SEM of four different experiments. VIP, vasoactive intestinal peptide, h.p.f., high power field.

Discussion

It seems certain that VIP induces eosinophil functional responses from the data presented in Fig. 1, but the dose–response curves are different from those induced by PAF, in which a specific cell-surface receptor has been identified. Although VIP modulates lymphocyte functions through specific lymphocyte receptors, which are typically seven transmembrane-coupled receptors, the VIP dose–response of eosinophils is in favor of non-specific binding activation. This is supported by: (i) lack of modulation of VIP-induced chemotaxis and degranulation by VIP receptor antagonist (4 Cl-D-Phe^{6}-Leu^{17}) VIP; (ii) lack of blocking activity of PTX in VIP-induced eosinophil chemotaxis; and (iii) lack of VIP-R1 expression by human eosinophils when compared to human lymphocytes. It is possible that VIP non-specific binding to human eosinophils may be due to either a non-specific charge-related binding or a lipophilic property. It is of note that other peptides, such as melittin and substance P, have been suggested to activate guinea-pig eosinophils through non-specific peptide–membrane phospholipid interaction; moreover, the MBP stimulates several cell types through negatively charged phospholipid binding sites.[28] Nonetheless, our failure to detect any signals for VIP-R1 mRNA in the gels does not solely exclude the possibility of expression of very low non-detectable amounts in the gel. In fact, only a few hundred protein molecules of G-protein-linked receptors are sufficient to mediate functional responses. In addition, the possibility of the existence of other receptors should be considered. The exact binding mechanism of VIP with human eosinophils remains of interest and requires further investigations.

Staurosporine and Ca^{2+} chelation did not modify VIP eosinophilotactic activity, which accordingly can exclude involvement of other pathways in VIP-induced chemotaxis, such as phospholipase A and phospholipase D, which are Ca^{2+} dependent.[29,30] However, modulation of tyrosine kinases and phosphatases by herbimycin A and pervanadate augmented and inhibited eosinophil chemotaxis, respectively. These interesting results suggest that the non-specific activation of human eosinophils by VIP seems to have two pathways: (i) a Ca^{2+}-dependent pathway involving EDN release; and (ii) a Ca^{2+}-independent tyrosine kinase and phosphatase pathway involving chemotaxis.

In vivo, allergic reactions result in the release of several mediators, many of which work in synergy, a phenomenon termed priming with respect to the *in vitro* studies. Therefore, we next investigated the priming activity of rhIL-5 and whether it cooperates with VIP in inducing significant eosinophil functional responses. Recombinant human IL-5 at 1 ng/mL did not induce any eosinophil chemotaxis or degranulation, but prepared the cell for an exaggerated response to subsequent stimulation with VIP, demonstrating a novel cytokine–neuropeptide interaction in inducing eosinophil functional responses. It is possible that rhIL-5 causes a change in the signal transduction pathway(s).

In conclusion, our findings suggest that VIP released in allergic reactions may act as a pro-inflammatory mediator, which exacerbates eosinophilic inflammation, rather than an anti-inflammatory mediator as a result of increase in cAMP concentration in other inflammatory cells, and this effect cannot be modulated by a receptor-specific antagonist.

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