

Downregulation of vasoactive intestinal polypeptide receptor expression in atopic dermatitis

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Background: Receptors for vasoactive intestinal polypeptide (VIP) have recently been suggested to play a key role in immunomodulation with genetically modified mice. However, it is not known whether changes in receptor gene regulation are involved in the pathogenesis of human immune disorders.

Objective: We studied the expression of VPAC₂ in acute lesions of the human immune disease atopic dermatitis.

Methods: By using nonradioactive in situ hybridization, quantitative immunohistochemistry, RT-PCR, and gene array studies, the expression status of VPAC₂ was assessed in atopic dermatitis and control tissues and in the human mast cell line HMC-1.

Results: In situ hybridization and immunohistochemistry demonstrated VPAC₂ mRNA and protein expression in human mast cells surrounded by VIP positive nerve fibers. Gene array experiments and RT-PCR studies showed high levels of VPAC₂ mRNA expression in mast cells that were increased compared to other receptors such as VPAC₁ or VIP in the human mast cell line HMC-1. Stimulation of HMC-1 cells led to a downregulation of VPAC₂. Similarly, quantitative immunohistochemistry for VPAC₂ in acute atopic dermatitis lesions showed a significantly decreased VPAC₂ immunoreactivity in mast cells.

Conclusion: The downregulation of VPAC₂ in human mast cells in acute lesions of atopic dermatitis suggests a role of this G-protein-coupled receptor in the pathophysiology of the disease. (*J Allergy Clin Immunol* 2003;111:1099-105.)

Key words: Mast cells, atopic dermatitis, VIP, neuropeptide, cytokine receptors

The function of most, if not all, cells in the mammalian organism is regulated by plasma membrane receptors of which the vast majority belong to the family of G-protein-coupled receptors, which are estimated to account for ~1% of all genes of the mammalian

Abbreviations used

AD: Atopic dermatitis

VIP: Vasoactive intestinal polypeptide

genome. Recently, the G-protein-coupled receptor VPAC₂ has been suggested to play a key role in immunomodulation with genetically modified mice.^{1,2} VPAC₂ recognizes the 28-amino acid protein vasoactive intestinal polypeptide (VIP) as specific ligand³ that has been demonstrated to act as neuromodulator with protective effects in a murine model of rheumatoid arthritis.⁴

To assess the pathophysiologic role of VPAC₂ in human immune disease, we addressed the regulation of VIP receptors in atopic dermatitis (AD), a common inflammatory skin disease with an onset mainly in early childhood, often representing the initial clinical manifestation of allergic disease and preceding allergic rhinitis, asthma, or food allergies, which we have recently characterized to be associated to heterogeneous genetic factors.⁵

In the human skin, mast cells are found particularly in association with structures such as blood vessels and nerves. They are bone marrow-derived, tissue resident cells, whose numbers are known to increase in a variety of inflammatory and neoplastic conditions. They also play a central role in the pathogenesis of AD and can be activated by a number of stimuli that are FcεRI dependent or independent. After activation, mast cells can immediately liberate mediators that induce immediate allergic inflammation. Activation may also be followed by the synthesis of chemokines and cytokines, and secretion of these mediators can contribute to chronic inflammation.⁶

Because it has previously been suggested that the interaction between mast cells and nerves in patients with AD is mediated by neuropeptides such as VIP,⁷ the present study was carried out to assess the role of the inducible VIP receptor VPAC₂ in human AD by using biopsies of acute AD lesions and cell cultures.

METHODS

Tissues and cells

In total, sections from 23 normal skin biopsies and 37 patients with AD were examined. AD diagnosis was based on the criteria of Rudzki et al,⁸ and routine histopathologic examination showed characteristic inflamed eczematous acute AD lesions. The human mast cell line HMC-1⁹ was provided by Dr J. H. Butterfield,

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Rochester, Minn, and the basophilic KU-812 cells¹⁰ were from the Borstel Research Institute, Borstel, Germany. Human skin mast cells were obtained from breast skin of patients ($n = 5$) undergoing cosmetic surgery or from noninflamed foreskin obtained from infants (age, 3 to 12 months; $n = 12$) by using a c-Kit antibody (YB5.B8, donated by L. Ashman, Adelaide, Australia) and magnetically cell sorting, as described,¹¹ resulting in $95\% \pm 4\%$ purity. All studies were performed according to the Declaration of Helsinki, after patients had given their informed consent.

Nonisotopic mRNA in situ hybridization

Sense and antisense digoxigenin-labeled VPAC₂ receptor specific cRNA probes (construct: pGEM-T vector) were generated as described previously,¹² and localization of cellular VPAC₂ receptor mRNA expression was examined by nonisotopic mRNA in situ hybridization by using a standardized protocol.¹² After permeabilization (0.1 mol/L HCl for 10 minutes), acetylation (0.1 mol/L triethanolamine [pH 8.0] containing 0.25% acetic anhydride for 10 minutes), dehydration, and incubation with prehybridization buffer (5 mmol/L EDTA, 10 mmol/L triethanolamine, 6.25% dextran sulfate, 0.3 mol/L NaCl, 1× Denhardt's, 1 mg/mL tRNA, 50% formamide), the sections were hybridized (temperature, 40°C) with hybridization buffer (containing 10 ng/μL VPAC₂ specific digoxigenin labeled sense or antisense probe in 50% formamide, 5 mmol/L EDTA, 1× Denhardt's, 10 mmol/L triethanolamine, 0.3 mol/L NaCl, 6.25% dextran sulfate, 1 mg/mL tRNA) for 14 hours. Hybridization was followed by RNase treatment, washing steps (2× SSC for 20 minutes at room temperature followed by 1× SSC for 20 minutes at room temperature, 0.5× SSC for 20 minutes at room temperature, 0.2× SSC for 20 minutes at room temperature, 0.2× SSC for 1 hour at 50°C, and finally 0.2× SSC for 15 minutes at room temperature) and detection with a digoxigenin detection kit (Boehringer Mannheim, Mannheim, Germany).

Cell cultures

HMC-1 cells were kept in Iscove's medium (GIBCO, Berlin, Germany), with 10% FCS (Seromed, Berlin, Germany) and 10⁻⁵ mol/L monothioglycerol (Sigma, Deisenhofen, Germany) added; KU-812 cells were kept in RPMI medium, with 20% FCS and 200 μmol/L mercaptoethanol. Cells were seeded into the different culture media at 1×10^5 cells/mL and fed every other day.

For mild stimulation to simulate the pathophysiologic situation, HMC-1 cells were treated for 4 hours with phorbol myristate acetate (PMA, Sigma) at a concentration of 1 ng/mL. RNA isolation for microarray analysis and RT-PCR were performed as described previously.¹¹

RT-PCR

The following sets of oligonucleotide primers were used to amplify cDNA: GAPDH: 5'-GATGACATCAAGAAGGTGGTG-3' and 5'-GCTGTAGCCAAATTCGTTGTC-3' (197 bp)¹³ and VPAC₂: 5'-CTGCACGGTGCCCTGCCCCAAAGT-3' and 5'-GCCCCCTCCACCAGCAGCCAGAAGA-3' (466 bp).¹⁴ Amplification was performed by using taq polymerase (GIBCO) over 24 to 38 cycles with an automated thermal cycler (Perkin Elmer, Ueberlingen, Germany). Each cycle consisted of the following steps: denaturation at 94°C, annealing at 58°C (reduced glyceraldehyde-phosphate dehydrogenase) and 67°C (VPAC₂), and extension at 72°C for 1 minute each. PCR products were analyzed by agarose gel electrophoresis and enzymatic digestion, with standard techniques.

Gene expression microarray analysis

After the demonstration of VPAC₂ mRNA and protein expression in situ, exploratory gene array studies were used to assess a possible

receptor regulation. The microarrays with stimulated and unstimulated HMC-1 cells were performed by Incyte Genomics and BioCat GmbH (Heidelberg, Germany). One hundred micrograms total RNA per sample was fluorescently labeled without any amplification of the RNA and hybridized on double spotted glass slides (Human UniGEM V 2.0 Microarray, BioCat). The chips were scanned with a 2 laser scanner, and the image was generated. The results were analyzed by the GEMtools analysis software (BioCat). The microarrays with RNA of stimulated and unstimulated cells were repeated once, and only values identical on both spots in both experiments were evaluated. Expression levels were measured as signal/background ratio with a cutoff value of 2.5 as described previously.¹⁵

Immunohistochemistry

Immunohistochemical assays were carried out as described previously¹⁶ by using cryostat or paraffin-embedded sections. After preincubations to reduce unspecific background signaling by using 4% bovine serum albumin in Tris-buffered saline, the sections were incubated with primary antibodies. For detection either the APAAP technique or fluorescent techniques with anti-mouse or anti-rabbit fluorescein-5-isocyanate antiserum (1:400; Amersham, Braunschweig, Germany) were carried out.¹⁷ For control, incubations without the primary antibodies or incubation with the VPAC₂ preimmune serum were carried out on parallel sections and did not lead to specific staining. The following antibodies were used: anti-mast cell tryptase (AA1; provided by A. Walls, Southampton, UK), anti-mast cell chymase (CC1; DAKO, Hamburg, Germany), anti-VPAC₂ antiserum (donated by E. J. Goetzl, San Francisco, Calif), and anti-VIP (Charles River, Southbridge, Mass, and Biogenesis, Poole, UK). For quantification of mast cell numbers, a standard protocol was used.¹⁸ In brief, randomized sections were evaluated by 2 independent investigators counting nucleated stained cells by using a raster covering 1/16 mm² at 1:400 magnification in at least 5 microscopic fields. Counts were expressed as stained cells per mm².

For quantification of VPAC₂ staining intensity in mast cells, a previously established protocol with an image analysis system (Optimas 6.5; Media Cybernetics, Bothell, Wash) was applied. Pseudo-color images of the stained cells were digitalized under constant staining and microscope settings, resulting in an intensity ranging from 0 to 255. Then, measurement of each mast cell staining intensity was performed for at least 4 slides of each patient and control subject.¹⁹ In addition, the staining intensity was scored manually by using a grade from 0 to 3 (absent, weak, moderate, strong) by 2 blinded observers.

Statistics

Results of the different parameters and groups are expressed as mean $\pm$ SEM. Statistical significance was assessed with the unpaired 2-tailed Student *t* test.

RESULTS

VPAC₂ expression in skin mast cells

Routine pathohistologic examination of skin biopsy specimens of 37 patients with AD showed characteristic inflamed eczematous acute lesions if compared to a control group ($n = 20$). Toluidine blue staining resulted in staining of mast cells in normal human skin, with a score of 35 ± 4 toluidine blue-positive mast cells per mm² and increased numbers of toluidine blue-positive mast cells (90 ± 8 per mm²) in AD sections (Fig 1). Incubation of serial sections with mast cell tryptase and chymase confirmed the increase of mast cells in AD tissues.

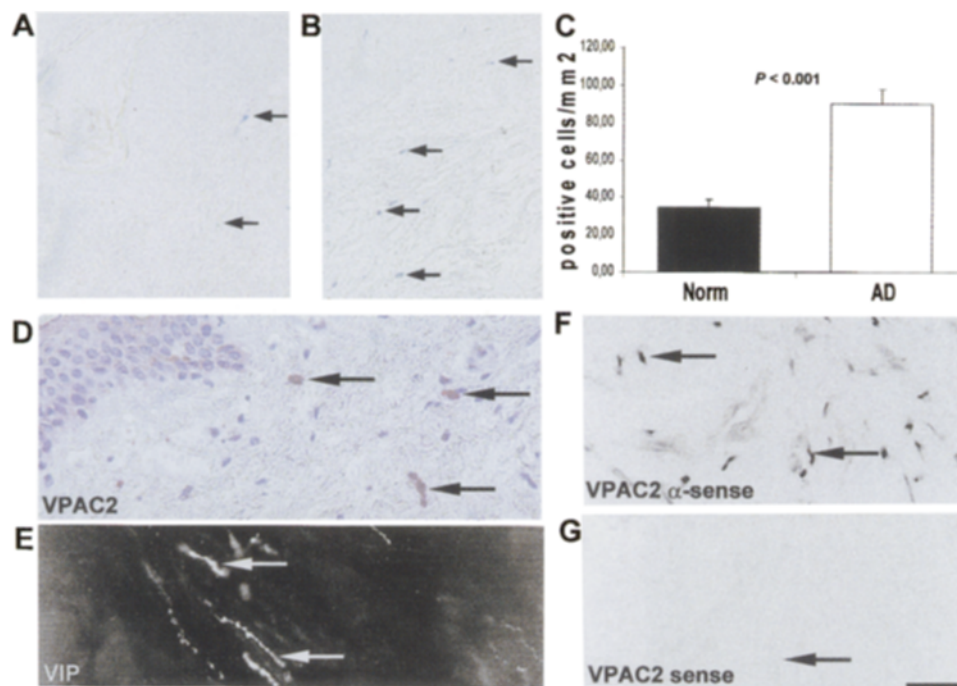


FIG 1. Mast cell numbers and VPAC₂ expression in AD. Sections of normal (A) and AD (B) human skin were evaluated for mast cell numbers, and toluidine blue staining showed an increased number of toluidine blue mast cells (arrows, 90 ± 8 per mm²) in AD tissues (C). VPAC₂ staining was found in mast cells (arrows in D), and signals for the ligand VIP were present in cutaneous nerve fibers (arrows in E). VPAC₂ mRNA in situ hybridization confirmed staining of mast cells (arrows in F). No specific staining was found when the sense probes were used (D). Scale bar represents 120 μ m (E = 60 μ m).

The expression of VPAC₂ in skin of AD patients and normal subjects was then examined by immunohistochemical analysis and nonradioactive in situ hybridization. Abundant staining of different cutaneous cell types was present in all areas of the biopsy specimens. Strongly positive VPAC₂ immunoreactivity and mRNA was found in the cytoplasm of mast cells (Fig 1), indicating a constitutive expression of VPAC₂ under basal conditions in human skin mast cells. VIP-immunoreactive nerve fibers were abundantly present in the papillary dermis (Fig 1) where they were associated with blood vessels and in close contact with infiltrating mononuclear cells. VPAC₂ mRNA hybridization signals were also present in hair follicle cells and sweat glands. Sense probe incubations did not lead to specific signaling (Fig 1).

Receptor expression in mast cells

After the demonstration of increased mast cell numbers in AD biopsy specimens and positive signals for VPAC₂ protein and mRNA in these cells, RT-PCR studies were carried out by using cutaneous mast cell preparations from mammary gland skin tissues or foreskin and HMC-1 and basophilic KU-812 cell lines to assess VPAC₂ mRNA expression under in vitro conditions. Repeated (n = 4) experiments showed abundant signaling in the different cell types; VPAC₂ was detected in breast skin and foreskin mast cells and in HMC-1 cells, but not in KU-812 cells (Fig 2).

HMC-1 gene array

After the demonstration of VPAC₂ mRNA and protein expression in situ, gene array studies were used as an exploratory approach to assess a possible regulation of the receptor by noxious stimulation in comparison to other receptors. Therefore, VIP receptor and VIP gene expression were examined in HMC-1 cells under basal and stimulated conditions. In comparison to genes of other receptors such as the gamma-aminobutyric acid A receptor with an expression factor value quantified as signal/background ratio of 2.5, VPAC₂ gene expression was markedly higher (56.3). Under stimulated conditions, the expression of VPAC₂ was downregulated from 56.3 to 30.4 (Fig 2).

Quantification of VPAC₂ protein expression in AD tissues

After the demonstration of VPAC₂ protein and mRNA expression in cutaneous mast cells and the finding of VPAC₂ mRNA downregulation in stimulated HMC-1 cells by using gene array studies, quantitative immunohistochemistry for VPAC₂ protein was applied to assess possible changes of mast cell-specific VPAC₂ expression on a translational level in situ in AD tissue (Fig 3). Scoring of intracellular staining intensity demonstrated a decrease of VPAC₂ protein in mast cells of the AD group (60.7 ± 14.0 SEM) when compared to normal subjects

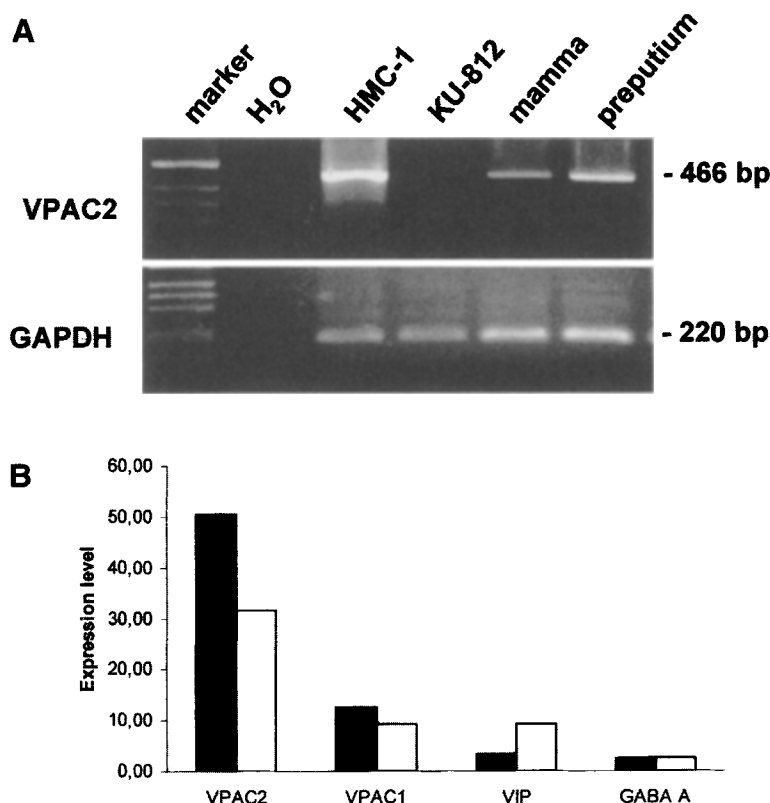


FIG 2. RT-PCR and gene array studies. Amplification products of the VPAC₂ gene were found in extracts of mammary gland skin (mamma) and foreskin (preputium) mast cell preparations and HMC-1 cells. There was no signal in KU-812 cells (**A**). The control gene was present in every preparation (reduced glyceraldehyde-phosphate dehydrogenase). HMC-1 cells were then examined for VPAC₂ gene expression under basal and stimulated conditions and showed high basal expression and a significant downregulation of VPAC₂ under stimulation in contrast to other receptors such as VPAC₁, GABA-A, or the ligand VIP (**B**).

(130.7 ± 18.7 , $P < .001$) (Fig 4). In addition, manual scoring confirmed (AD, 1.3 ± 0.3 ; normal, 2.8 ± 0.4 ; $P < .01$) these results.

DISCUSSION

The present studies addressed the issue of a possible regulation of G-protein-coupled VIP receptors in human immune disease by using AD as a model disease because VIP is widely expressed in the skin.^{20,21} Here it serves as mediator of a multitude of biologic functions including neurotransmission, control of regional blood flow, and immune response.²²

Two molecularly distinct VIP receptors have been cloned and characterized in past years. In addition to the VPAC₁ receptor, which was identified in central nervous system, lung, and other tissues,²³⁻²⁵ a second receptor that responds to VIP and PACAP with comparable affinity was characterized in rat,^{25,26} mouse,²⁷ and human tissues.^{28,29} Among cutaneous cells, messenger RNA of VPAC₂ was found in extracts of HaCaT cells¹⁴ and localized to immune cells, keratinocytes, and endothelial cells.²⁰

Recently, a crucial role of the VPAC₂ receptor in immunomodulation was demonstrated in genetically modified murine models. It was shown that transgenic mice with constitutive T-cell expression of VPAC₂ have significant elevations of blood IgE, IgG1, and eosinophils. These mice also displayed a depressed delayed-type hypersensitivity.² Parallel studies with a model of mice lacking the inducible VPAC₂ receptor demonstrated an enhanced delayed-type hypersensitivity and diminished immediate-type hypersensitivity, indicating a potential role of VPAC₂ in human immune diseases.¹ The present study was carried out to assess the role of VPAC₂ in a human immune disease by using skin biopsy specimens of acute AD lesions and control biopsy specimens and led to the demonstration of an inverse correlation between receptor expression and immune disease in mast cells, which have previously been shown to play a major role in AD and are increased in AD skin lesions as shown here and previously.³⁰

Histopathologically, acute eczematous skin lesions as examined here are characterized by an epidermal intercellular edema and the presence of antigen-presenting cells such as Langerhans' cells and macrophages with

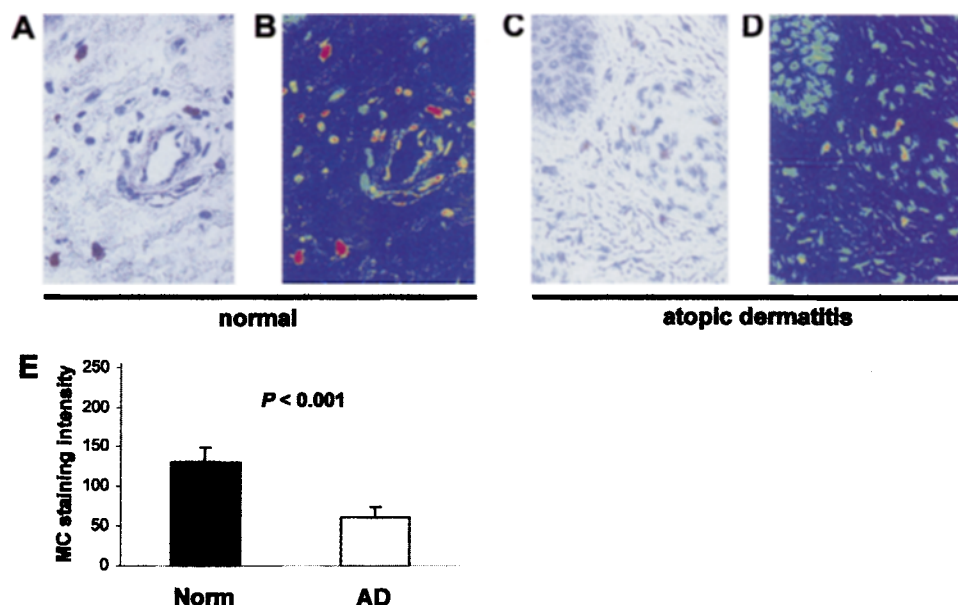


FIG 3. Mast cell VPAC₂ protein levels in situ. Staining intensity of VPAC₂ protein was analyzed in mast cells of normal subjects (A and B) and AD patients (C and D) by using image quantification methods, constant staining and microscope settings, and pseudo-color imaging (B and D). Staining intensity that was measured in single mast cells (MC) was decreased in AD if compared to normal individuals (E). Scale bar represents 100 μ m.

IgE molecules. Also, mast cell and CD4-activated T-cell numbers are elevated. In the present study, acute lesions were examined because these lesions displayed the closest proximity to the previously used animal model of cutaneous hypersensitivity, which suggested a participation for VPAC₂.^{1,2}

The precise mast cell mediators involved in AD have not been determined so far. Although histamine is the crucial mediator of pruritus in type I allergic reactions such as urticaria, it does not play a major role in the pathogenesis of AD; thus, other mediators must be involved.³¹ The present finding of an impaired VIP receptor expression in mast cells of AD patients, as quantified in situ and validated by in vitro studies with a low concentration of PMA to mildly stimulate the mast cells and microarray techniques, suggests that an imbalance of neuroimmune interactions may partly account for the development of the disease.

The use of PMA in a low concentration to stimulate the cell line was presently used to validate the in situ data. Whereas it is clear that there could be more physiologic stimuli such as cytokines or other mediators, this tumor promoter was presently chosen as classic stimuli to mimic a noxious background as found in acute lesions. Future studies with a variety of different mediators such as cytokines or chemokines could show the effect of each of these mediators on HMC-1 cell function and gene expression.

A link between the nervous system and the inflammatory cutaneous processes occurring in AD has been postulated before because erythema and pruritus can be induced by several neuropeptides. Furthermore, decreased reac-

tions in AD to some neuropeptides including VIP have been suggested to be due to an increased local availability of the substances. In fact, elevated skin levels of VIP have been reported,³² although others have failed to demonstrate this mediator in suction blister fluid.³³ A further finding that may speak against an upregulation of the ligand VIP is the absence of increased VIP-immunoreactive nerve fibers in AD skin.³⁴

The present data show that VIP effects in immunologic diseases may not only be modulated by the amount of secreted VIP but also by the regulation of VIP receptor expression, because mast cell-specific expression of VPAC₂ is downregulated in AD.

A direct modulatory effect of VIP on mast cells has previously been suggested,³⁵ but distinct mast cell receptors for VIP have not been found in situ. VIP has been shown to act predominantly via stimulation of adenylyl cyclase and cyclic adenosine monophosphate (cAMP) production, and studies on VIP gene expression have demonstrated a 2-way relationship between stimulation of VIP synthesis and high levels of cAMP.³⁶ When cAMP levels decrease in mast cells, degranulation increases.³⁷ An elevated cAMP production activates cAMP- and cyclic guanosine monophosphate-dependent protein kinases,³⁸ and VIP might thus lead via this VPAC₂-related cascade to an inhibition of cutaneous mast cell secretion. In this respect, the presently demonstrated downregulation of VPAC₂ in mast cells of AD patients may account for reduced protective and stabilizing effects of VIP, which are found under normal conditions and therefore may promote accelerated mast cell degranulation in AD.

An important issue to address the role of VPAC₂ in AD arises from the question whether the downregulation of VPAC₂ is an epiphenomenon resulting from the effects of other mediators such as cytokines. So far, the only previous indication on the possible involvement of the receptor was found by using 2 animal models with transgenic VPAC₂ overexpression and VPAC₂ gene depletion. These studies reported a state of enhanced hapten-evoked cutaneous delayed-type hypersensitivity and depressed immediate-type hypersensitivity after VPAC₂ gene depletion. These data indicate that the loss of function of VPAC₂ could be by itself related to an allergic diathesis of animals unaffected by other mediators. However, in the present studies, it could not be distinguished whether the downregulation of VPAC₂ in acute AD lesions is due to the effects of other mediators (ie, IgE cross-linking), and future functional studies may address this question. In this respect, the only available data on VIP receptor downregulation by mediators have been reported for in vitro stimulation of T-helper cells with soluble anti-CD3 plus PMA, which induced a T-cell activation-dependent downregulation of VPAC₁. It was also shown that VPAC₂ mRNA content decreased in granuloma cells from IL-4 mutant mice infected with *Schistosoma mansoni*, when cultured with rIL-4.

We have previously demonstrated that VPAC₂ mRNA is localized not only to inflammatory cells such as mast cells but also to a variety of other cells including keratinocytes with a signal intensity that was maximal at the basal zone and decreasing to superficial layers, endothelial cells, or to cells of eccrine sweat glands and to cells of the germinative epithelium, medulla, and matrix of the hair follicle.^{20,21} In view of recent data on the relevance of keratinocytes in inflammatory changes in AD,³⁹ it could be speculated that VPAC₂ may also be regulated in these cells. However, a recent report on the regulation of VPAC receptors in human keratinocytes demonstrated that the predominant keratinocyte receptor regulating VIP effects appears to be VPAC₁ but not VPAC₂.⁴⁰

In addition to AD, there are other immune diseases in which this neuropeptide might play a major role. In this respect, a recent study has assessed the role of VIP in a mouse model of rheumatoid arthritis. It was demonstrated that VIP significantly reduced the incidence and severity of arthritis in an experimental model, completely abrogating the destruction of joint and bone structures.⁴ In contrast to the present study, in the animal model of rheumatoid arthritis the effects of VIP were pharmacologically mediated via the VPAC₁ receptor because a VPAC₁, but not a VPAC₂, agonist was able to mimic the protective effects of VIP.

In conclusion, the transcriptional and translational mast cell-specific downregulation of VPAC₂, as shown here in vitro and in vivo, adds an important dimension to the understanding of cellular pathophysiology underlying immune diseases and suggests that the downregulation of VPAC₂ in patients with AD may contribute to the pathophysiology of the disease.

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