

## VASOACTIVE INTESTINAL PEPTIDE, PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE AND IMMUNE SYSTEM: FROM BASIC RESEARCH TO POTENTIAL CLINICAL APPLICATION

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*Vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP) are important immune mediators sharing cellular origin, receptors and functions affecting both the innate and the acquired immunity. VIP and PACAP have been clearly identified as potent antiinflammatory factors, which act by regulating the production of both anti- and pro-inflammatory mediators. In this context, both peptides have been described to prevent death by septic shock, an acute high mortality inflammatory disease. Additionally, VIP and PACAP regulate the expression of costimulatory molecules, an action that may be related to modulating the shift toward Th1 and Th2 differentiation. We have recently reported that both peptides prevent the deleterious effects of an experimental model of rheumatoid arthritis, by downregulating both inflammatory and autoimmune components of the disease. Thus, VIP and PACAP represent potential multistep therapeutic agents that provide the mechanisms to act at different levels in the complex network cascade formed by chemokines and cytokines involved in the regulation of inflammatory/Th1 diseases. Based on the protective effects of VIP and PACAP we conclude that the exogenous administration of these peptides could offer an alternative to existing treatments for arthritis and other inflammatory/Th1-autoimmune diseases, such as multiple sclerosis, inflammatory bowel disease, or autoimmune diabetes, as well as endotoxic shock.*

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### INTRODUCTION

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Health is the result of regulatory interactions between three systems involved in organism homeostasis: the nervous, endocrine and immune systems. Extensive research of the complex network formed by these three systems, over the past 20 years, has resulted in a now well established area of research: the neuroimmunomodulation. A crucial factor for

the functioning of this network was the demonstration that cells of nervous, endocrine and immune systems synthesize and secrete similar substances, bearing the same receptors for them and reducing the previously established differences between neurotransmitters, hormones and immune mediators (1). Initially it was established that neurons and endocrine cells possessed similar substances; only later was it demonstrated that cells of the immune system were also involved in

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production of these same agents. In this sense, the first contributions made in 1980, showed that macrophages and lymphocytes were able to produce ACTH and endorphins (2). Today, 27 neuroendocrine mediators that could be produced by lymphoid cells have been described. In the last years, the study of these common neuro-endocrine-immune mediators has undergone a striking growth, not only in basic research. Rather, their potential clinical application is now becoming a reality.

In this review, we will discuss some aspects of the immunobiology of vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP), as well as suggest a potential significant therapeutic role for these peptides in inflammatory and autoimmune diseases.

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#### LYMPHOCYTES ARE THE IMMUNE SOURCE OF VIP AND PACAP

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VIP and PACAP belong to the glucagon-growth hormone releasing factor-secretin superfamily of peptides found in nervous, endocrine and immune systems of vertebrates. Family members show a highly conserved sequence, similar gene structural organization and a great degree of homology between the peptides and their precursors. In 1969 Said and Mutt (3) reported for the first time to the scientific community the presence, in the lung, of VIP, a 28-aminoacid peptide that was isolated from the small intestine (4) as a gastrointestinal hormone. VIP was later described in the central and peripheral nervous system, acting as a neurotransmitter and neuromodulator, and is today considered an important immunomodulator. In the immune system, VIP is produced in single and double positive (CD4+CD8+) thymocytes, and in T and B lymphocytes from spleen and lymph nodes and is released to the lymphoid microenvironment after treatment with agents that mediate important immune functions, such as proliferation and antigen stimulation, inflammation or apoptosis (5-7). Twenty years later PACAP was discovered (8), becoming most recent member of the VIP-glucagon-growth hormone releasing factor-secretin superfamily. Initially isolated from ovine hypothalamic extracts, based on its ability to activate cAMP formation in anterior pituitary cells, it exists in two amidated forms, PACAP27 and PACAP38, with 27 and 38 amino acid residues, respectively. The PACAP is widely distributed throughout the organism, located in nerve cell bodies and nerve fibers of the central and peripheral nervous system and is especially abundant in the central nervous system, adrenal medulla and testis (9), performing diverse biological activities (reviewed in 10, 11). Similar to VIP, PACAP was later "rediscovered" in the immune system and demonstrated to be produced in central and peripheral lymphoid organs (12,13). Additionally, similar to other peripheral tissues where both VIP and PACAP appear to be coexpressed in the same cells,

PACAP storage and gene expression in central (thymus) and peripheral (spleen and lymph nodes) lymphoid organs appears exactly in the same lymphocyte subpopulations as VIP. Thus VIP and PACAP are coexpressed in single and double positive (CD4+CD8+) thymocytes and in T and B lymphocytes from spleen and lymph nodes (13). There are few differential functions between VIP and PACAP in the immune system, since most of the actions exerted by VIP are also shared by PACAP. Thus VIP, together with PACAP, are two multifunctional and pleiotropic signal molecules of the neuro-endocrine-immune network that exert important actions related to the innate and acquired immunity (see also this volume of *Biomedical Reviews*, Meeting Abstracts, pages 66-68).

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#### THREE VIP/PACAP G-PROTEIN COUPLED RECEPTORS

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VIP and PACAP exert numerous biological functions through binding to specific receptors, that together with the receptors for VIP-related peptides constitute an original subfamily within the superfamily of G-protein coupled receptors (14). This subfamily, referred to as class II, also comprises receptors for parathyroid hormone, calcitonin, corticotropin-releasing factor, and the so-called EGF-TM7 receptors (15). Class II subfamily of receptors display several common properties including large N-terminal extracellular domains containing highly conserved cystein residues, N-terminal leader sequences, and complex gene organization with many introns (16). The N-terminal domain plays an important role in the binding of the ligand (16-18), although both extracellular and transmembrane domains are also involved (19-21). To date, three receptors have been described in vertebrates with a wide distribution in different cell types, including cells of nervous, endocrine and immune systems: VPAC1, VPAC2 receptors that bind VIP and PACAP with equal affinity, and the PAC1 receptor that is PACAP selective (11). In the immune system, we have extensively studied the expression and distribution of these three receptors in cells of central and peripheral lymphoid organs.

The VPAC1 receptor was the first VIP receptor described and cloned (22) and binds VIP and PACAP with equal affinity. To date, no splice variants of this receptor have been described. This receptor is coupled mainly to the adenylate cyclase (AC) pathway. In the immune system, the VPAC1 receptor was identified for the first time in 1981 using binding techniques in human peripheral blood lymphocytes (23). Later, it was reported in human monocytes (24), murine lymphocytes (25,26) and rat alveolar macrophages (27,28). Additionally, gene expression of VPAC1 receptor has been demonstrated in T and B murine lymphocytes subpopulations from peripheral lymphoid organs, in double and single positive CD4+CD8+ murine thymocyte subsets and peritoneal macrophages (29-31). As it will be discussed below, it is the main receptor invol-

ved in the beneficial role exerted by VIP and PACAP in inflammatory and autoimmune diseases.

The VPAC2 receptor was described for the first time in the human lymphoma cell line SUPT1 (32) as a VIP/helodermin preferring receptor and cloned for the first time from the rat olfactory bulb (33). To date, no splice variants of this receptor have been described. Similar to VPAC1, VPAC2 binds VIP and PACAP with comparable affinity, but does not bind secretin with as high affinity as does the VPAC1 receptor. The VIP/PACAP effects exerted through interaction with VPAC2 receptor are mediated by the AC pathway. In the immune system, its expression has been reported in lymphocytes and macrophages, but it is inducible, being detected in lymphocytes only after stimulation through the T cell receptor (TCR) associated CD3 molecule, and in macrophages after lipopolysaccharide (LPS) stimulation (34,35). VPAC2 receptor is detected in mononuclear cells by immunohistochemical techniques two days after the detection of VPAC1 receptor, at sites of inflammation and antigen recognition (36). However, constitutive expression of VPAC2 receptor has been reported in human lymphoid cell lines (32,37).

The PAC1 receptor is a PACAP selective receptor but in micromolar concentration VIP is a heterologous ligand (14). It has eight variants produced by alternative splicing of the transcript that codifies to N-terminal (38), IC3 (39,40) and TM-2 and TM-4 (41), that are involved in ligand binding, activating both AC and inositol triphosphate/phospholipase C (IP/PLC) pathways as well as the activation of an L-type calcium channel, respectively. Recently, a novel splice variant in the C-terminal domain of frog PAC1 receptor was described (42). Given the wide range of splice variants described for PAC1, there are still several possibilities that remain to be elucidated regarding ligand binding or coupling to second messengers. In immune cells, this receptor is expressed only in macrophages, binding VIP and PACAP with the same affinity and activating the IP/PLC pathway (43). Lymphocytes lack PAC1 receptor expression. Although it is still unknown whether the splice variant of PAC1 is present in macrophages, it seems clear that this receptor plays a crucial role in the VIP and PACAP regulation of several agents involved in inflammation, such as IL-6 (44). Thus, the redundancy between VIP and PACAP, in terms of synthesis and effects in the immune system, is also described at the receptor level, explaining in part the pivotal role attributed to both neuropeptides in the control of immune homeostasis and disease.

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#### VIP/PACAP AGENTS AGAINST INFLAMMATION

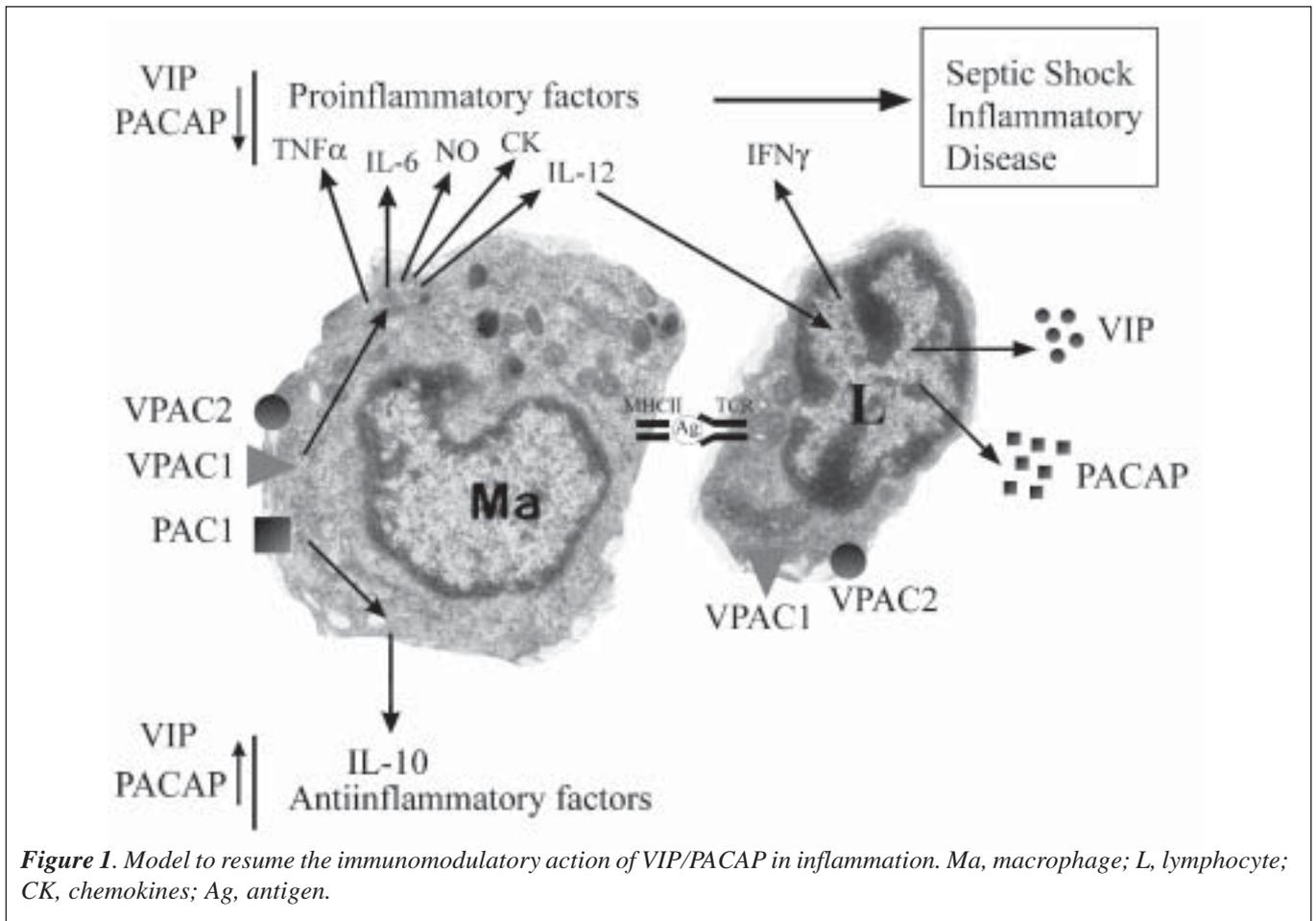
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Inflammation is a vital process that involves both nonantigen specific and antigenspecific mechanisms. The two main cell types involved in these mechanisms are macrophages and lymphocytes. Macrophages play a crucial role against patho-

gens, by contributing to integrate both non-antigen and antigen-specific defense mechanisms. Phagocytosis of pathogens is the main characteristic of macrophages, leading to their activation in terms of cytokine production and antigen presentation and to the reduction of the pathogen load. Macrophages initiate the inflammatory response through the secretion of inflammatory cytokines and production of reactive oxygen and nitrogen intermediates. Additionally, antigen specific mechanisms are based on clonal activation of T and B lymphocytes that is triggered in cooperation with both antigen specific and nonspecific cell populations. Signals from accessory populations include antigen presentation and mediators, such as cytokines, that lead to activation, proliferation and differentiation of lymphocytes.

VIP and PACAP have been identified as potent antiinflammatory factors, which act by regulating the production of both anti- and proinflammatory mediators. In addition, VIP and PACAP modulate the expression of costimulatory molecules and in this manner may function as modulators of Th1/Th2 differentiation. Microbial products, such as LPS, induce macrophages to secrete several proinflammatory products such as tumor necrosis factor-alpha (TNF- $\alpha$ ), interleukin-12 (IL-12), IL-1, IL-6 and nitric oxide (NO). VIP and PACAP inhibit the production of these proinflammatory mediators and stimulate the production of the antiinflammatory cytokine IL-10 in macrophages activated by microbial products or interferon-gamma (IFN $\gamma$ ) (45-49) (Fig. 1). These effects are mediated by the presence of PAC1, VPAC1, and VPAC2 receptors on activated macrophages. Additionally, macrophages participate in the antigen specific responses, acting as antigen presenting cells and providing T lymphocytes with costimulatory molecules and a cytokine environment that influences the proliferation and differentiation of the T cells. Among the costimulatory molecules, B7.1 and B7.2 play major roles and are expressed in macrophages only following activation, with B7.2 being induced earlier and at higher levels than B7.1 (50). VIP and PACAP are endogenous factors that regulate B7 expression in macrophages. Interestingly, these peptides affect B7 expression in resting and activated macrophages in an opposite manner. In resting macrophages, PACAP upregulates B7.2, but not B7.1 expression at mRNA and protein level, both *in vivo* and *in vitro*. In contrast, in LPS/IFN $\gamma$ -activated macrophages, PACAP downregulates both B7.1 and B7.2 expression. The effects of VIP and PACAP on B7 expression correlate with effects on the stimulatory activity for T cells. The inhibition of B7.1/B7.2 expression in activated macrophages is in perfect agreement with the accepted role of VIP and PACAP as endogenous antiinflammatory agents.

VIP and PACAP not only modulate macrophage function but also exert important immunomodulatory actions in lymphocytes. Following antigenic stimulation, CD4 T cells differentiate into Th1 and Th2 effector cells, characterized by spe-



cific cytokine profiles and functions. Determining factors for their differentiation include the nature of the APC and the expression of the costimulatory molecules expressed by them and in the cytokine microenvironment (reviewed in 51). Among cytokines, IL-12 and IL-4 appear to be the determinant factors for Th1 and Th2 differentiation, respectively. Regarding to the immunomodulatory role of VIP and PACAP in Th1/Th2 differentiation, it has been shown that these peptides induce Th2 responses *in vivo* and *in vitro* (52). Macrophages treated *in vitro* with VIP and PACAP are able to induce Th2- type cytokines (IL-4 and IL-5) while inhibiting Th1-type cytokines (IFN $\gamma$ , IL-2) in Ag-primed CD4 T cells. *In vivo* administration of VIP and PACAP in Ag-immunized mice result in a decreased number of IFN $\gamma$ -secreting cells and an increased number of IL-4 secreting cells (52). There are several possible non-excluding mechanisms for the VIP and PACAP bias towards Th2. Inhibition of macrophage IL-12 production by PACAP is one possibility. Since IL-4 dominates over IL-12, driving naive CD4 T cells toward the Th2 phenotype (51), a reduction in IL-12 by PACAP, even in the absence of an effect on IL-4, will result in Th2 differentiation. A

second possibility is upregulation of B7.2 expression on macrophages by PACAP. In view of these data demonstrating VIP and PACAP modulation of several crucial steps of natural and acquired immunity, it was tested whether these peptides could be used as agents to ameliorate or prevent several acute and chronic inflammatory and autoimmune disorders.

#### VIP AND PACAP AS POTENTIAL THERAPEUTIC AGENTS IN INFLAMMATORY AND AUTOIMMUNE DISORDERS

Different animal models have been used to study the potential therapeutic effects of VIP and PACAP in human diseases, such as septic shock, rheumatoid arthritis (RA), and Crohn's disease that share similar characteristics in terms of participation of (i) proinflammatory cytokines, (ii) oligoclonal expansion, and (iii) activation of CD4+ T cells with exacerbate Th1 production of cytokines.

#### Endotoxic shock

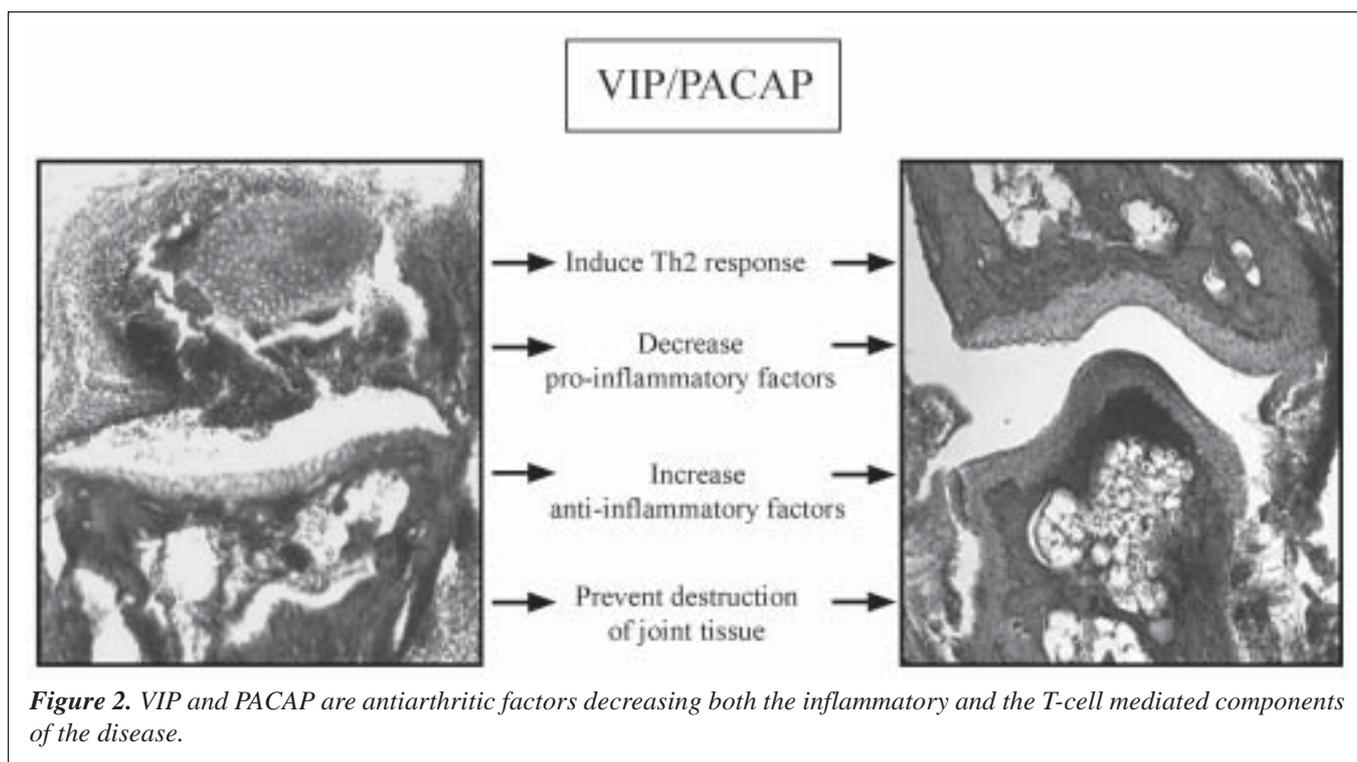
Although the inflammatory process is a localized protective response, the sustained production of inflammatory mediators

leads to serious pathological conditions, such as endotoxic shock. Septic shock, the common cause of death in the intensive care unit, is a systemic response to severe bacterial infections, generally caused by Gram-negative bacterial endotoxins that induce the generation of proinflammatory factors (53). Because of their antiinflammatory properties, VIP and PACAP have been reported to protect against endotoxic shock syndrome (54). TNF- $\alpha$  and IL-6 are overproduced in this state and VIP and PACAP have been shown to reduce the circulating levels of these cytokines in an animal model of such pathology (55). Exogenous administration of VIP and PACAP protect mice from the lethal effects of high endotoxemia, by downregulating proinflammatory mediators such as TNF- $\alpha$ , IFN $\gamma$ , IL-6, IL-12, and NO (54). Although our *in vitro* and *in vivo* studies using specific VIP agonists have indicated that the VPAC1 receptor is the main player of the VIP and PACAP antiinflammatory action (56), we have recently demonstrated, using knockout mice for the PACAP receptor, PAC1 receptor involvement (44). Our results indicated that PAC1 receptor acts *in vivo* as an antiinflammatory receptor, at least in part, by attenuating LPS-induced production of proinflammatory IL-6, which appears to be the main cytokine regulating the expression of the majority of acute phase protein genes, important deleterious components of septic shock. Additionally, we have shown PAC1 receptor involvement in the inhibition of neutrophil infiltration, measured both by myeloperoxidase activity and microscopic analysis (57) in different key organs affected by endotoxemia, such as liver, lung and bowel. VIP and PACAP have been demonstrated to protect from endotoxemia if given two hours after endotoxin injection (55) probably due to the pleiotropic effects inhibiting proinflammatory mediators that appear later during the inflammatory response.

### **Rheumatoid arthritis**

Given that VIP and PACAP provide the mechanisms to mediate the complex network cascade formed by chemokines and cytokines involved in the regulation of inflammatory/Th1 diseases, we next evaluated the potential therapeutic role of both peptides in a collagen-induced model of arthritis. Rheumatoid arthritis is a chronic and debilitating autoimmune disease of unknown etiology that leads to chronic, progressive inflammation in the joints and subsequent erosive destruction of the cartilage and bone. The main symptoms of this disease result from a massive infiltration of immune cells such as neutrophils, macrophages and T cells into the synovial membrane and fluid. These immune cells, together with activated synoviocytes, release high amounts of chemokines which recruit cells to the site of inflammation, matrix metalloproteinases (MMP) which destroy the joint tissues, and proinflammatory mediators as TNF- $\alpha$ , IL-1, IL-6, and IFN $\gamma$  which contribute to generate the joint damage (58). Moreover, although the con-

tribution of Th1 and Th2 responses in RA is not completely understood, several studies in animal models revealed that Th1 cytokine profile predominates at the induction and acute phases of the disease, whereas Th2-mediated responses are associated with the remission phase of the disease (59-63), thus suggesting a pathogenic role of Th1-derived cytokines. In RA, the synovium, which under normal conditions is a fragile bilayer membrane covering the cartilage and bone in the joint, is transformed into a thick invasive one that destroys the joint structure, producing deformation of the tissue, explaining, in part, the rigidity and paralysis of the patients in the last phases of the disease. The experimental murine model of collagen-induced arthritis is produced by immunization with type II collagen and shares common clinical, histological, and immunological features with human RA. We have shown, using this model, that treatment with VIP and PACAP produce a general beneficial amelioration of the disease (64-65). Treatment of arthritic mice with VIP and PACAP decreases the frequency of arthritis, delays its onset, reduces the severity of symptoms and prevents joint damage. The therapeutic effects of both peptides is due to a reduction of the two deleterious components of the disease, the inflammatory and the autoimmune (Fig. 2). Thus, VIP and PACAP not only produce a reduction in the levels of proinflammatory agents, such as TNF- $\alpha$ , IL-6, IL-1 $\beta$ , IL-12, iNOS and IL-18, but also increase the levels of antiinflammatory cytokines, such as IL-10 and IL-1Ra. VIP and PACAP also downregulate the levels of chemokines (RANTES, MCP-1, MIP-1 $\alpha$ , and MIP-2) responsible for the infiltration and activation of various leukocyte populations in joint tissue and which contribute to the pathology of RA. Moreover, VIP and PACAP also reduce the expression and activity of some MMP, which have a crucial role in the depletion of proteoglycan and contributing to the destruction of both cartilage and bone. Because of the decreasing levels of all of these harmful soluble factors in RA, there is a patent remission of the chronic inflammation of the joints of the affected mice. The effects of both peptides are not restricted to the mediators produced by synoviocytes, but also affect the cytokines released by the infiltrated T cells. There is strong evidence that the majority of the T cells in the inflamed tissues in RA have a Th1 cytokine pattern (59). Additionally, the Ig isotype switching that is directed by Th1 or Th2 cytokines in a different way (IFN $\gamma$  and IL-4 induce IgG2a and IgG1 synthesis, respectively) is another marker of this disease that shows high anti-type II collagen IgG2a circulating levels (66). VIP and PACAP treatments produce a reduction of IFN $\gamma$  levels (Th1 cytokine) and an increase of IL-4 levels (Th2 cytokine) together with a reduction of IgG2a and an increase of IgG1. These two effects confirm that VIP and PACAP induce a Th2 response, possibly contributing to the remission of the illness and blocking the autoimmune component of this disease. Of biological significance is the fact that VIP levels, similar to those of other recently described



antiarthritic neuropeptides and hormones, such as calcitonin gene-related protein and melanocyte-stimulating hormone (67-68), are specifically increased in serum and joints of arthritic mice during the development of the disease. This fact suggests that endogenous neuroimmune mediators represent a natural antiarthritic machinery activated in response to autoimmune/inflammatory conditions, such as arthritis, in an attempt to counterbalance the effects of inflammatory mediators. VIP and PACAP affect both the inflammatory and autoimmune components of the disease, representing a new approach over already existing treatments of RA.

#### CONCLUSION

VIP and PACAP represent potential multistep therapeutic agents that provide the mechanisms to act at different levels in the cascade of the complex network formed by chemokines and cytokines involved in the regulation of inflammatory/Th1 diseases. Based on the protective effects of VIP and PACAP, the exogenous administration of these peptides could offer an alternative to existing treatments for arthritis and other inflammatory/Th1-autoimmune diseases, such as multiple sclerosis, inflammatory bowel disease, or autoimmune diabetes, as well as for endotoxic shock.

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