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VIP and PACAP as Regulators of Immunity: New Perspectives from A Receptor Point of View

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Abstract

Vasoactive Intestinal Peptide (VIP) and Pituitary Adenylate Cyclase-activating Polypeptide (PACAP) are two neuropeptides acting through three common G-protein coupled receptors (VPAC1, VPAC2 and PAC1). Among their pleiotropic actions within the organism, VIP and PACAP are known to exhibit immunomodulatory properties in both the innate and adaptive immune axes. The fact that they inhibit inflammation in murine models of disease has brought these peptides into the spotlight within the field of therapeutic discovery for autoimmune/inflammatory diseases. Pharmacological tools and transgenic mice have been useful in order to investigate the involvement of each of their three receptors in these actions. This review focuses on the relevance of the VPAC2 receptor on VIP and PACAP modulation of immune responses, and discusses its potential as a target for the treatment of Th1-driven inflammatory disorders.

VIP and PACAP

VIP (vasoactive intestinal peptide) and PACAP (pituitary adenylate cyclase-activating polypeptide) belong to a superfamily of structurally related peptides including secretin and glucagon. Whereas VIP is a 28 aminoacid peptide, PACAP can be found in two amidated forms of 27 (PACAP27) or 38 (PACAP38) aminoacids. Because these peptides exhibit a high sequence and structural homology (i.e. 68% identity between VIP and PACAP27), it has been proposed that their genes derived from a common ancestral gene subjected to duplication and divergence during the course of evolution [1-3]. VIP and PACAP were originally isolated from the small intestine and the pituitary, respectively, although it has been later demonstrated that they are widely distributed in the organism [4,5]. The fact that their primary structures have been well conserved in vertebrates suggest that they play important physiological actions. In fact, they modulate multiple processes of the digestive, respiratory, reproductive and cardiovascular systems among others. VIP and PACAP act through three G-protein coupled receptors (GPCRs) named VPAC1, VPAC2 and PAC1 [6]. Whereas VPAC1 and VPAC2 bind both VIP and PACAP with equal high affinity, PAC1 exhibits 100 to 1000 times higher affinity for PACAP than for VIP [1]. Their main signaling pathway involves adenylate cyclase activation through accessory G-proteins and cyclic AMP (cAMP) synthesis. Nevertheless, activation of other signaling pathways involving phospholipase C (PLC) or phospholipase D (PLD) or intracellular calcium increases has been also reported [7]. An overview of the roles of one of the VIP and PACAP receptors, the VPAC2 receptor within the immune system compartment is discussed in the present review and is illustrated in the figure 1.

The VPAC2 Receptor

VPAC2 was first cloned by Lutz et al. [8] from rat olfactory bulb. Mouse and human VPAC2 were subsequently cloned from insulin-secreting beta-cell line MIN6 and SUP-T1 lymphoblast libraries, respectively [9]. Its gene Vipr2 maps to the rat chromosome 4, the mouse F2 region of chromosome 12 and human chromosomal region 7q36.3 [10,11]. Regarding its distribution, in the central nervous system, its highest expression is found in the suprachiasmatic nuclei, where it modulates circadian rhythms [12], but it is also present in the thalamus, hypothalamus, midbrain and brainstem. In the periphery, a systematic study in mice revealed its expression in the smooth muscle of blood vessels and gastrointestinal and reproductive systems, lung, colon, kidney, adrenal medulla, retina and pancreas [13].

Based on its protein structure, VPAC2 belongs to the class B GPCR family receptors, which exhibit seven transmembrane domains and a series of common features such as a large N-terminal (Nter) ectodomain containing several N-glycosylation sites and six highly conserved cysteine residues forming three disulfide bridges, and a signal peptide for addressing the receptor towards the plasma membrane. Multiple structure-activity relationship studies from several research groups have shown that in all class B GPCRs, the large Nter domain is critical for ligand recognition; mostly due to the presence of a Sushi domain which is characterized by two antiparallel β sheets and stabilized by three disulfide bonds and by a salt bridge between acidic and basic residues.

Based on (1) photoaffinity experiments which identified four physical interaction sites between VIP and the Nter domain of VPAC1 receptor, (2) the NMR structure of the ligand VIP which revealed mostly an alpha helical structure, and (3) the 3D model of the VPAC1 N terminal domain comprising a Sushi domain [14,15], an accurate 3D model illustrating the interaction between the VIP molecule and the Nter domain of the VPAC1 receptor has been created. This model suggested that the C-terminal and central α-helical parts of the VIP peptide interact with the VPAC1 Sushi domain [14-17]. Consistent with a two-site binding model, it has been speculated that this may bring the N-terminus of the peptide into the appropriate position to contact the transmembrane region of the receptor leading to its subsequent activation.

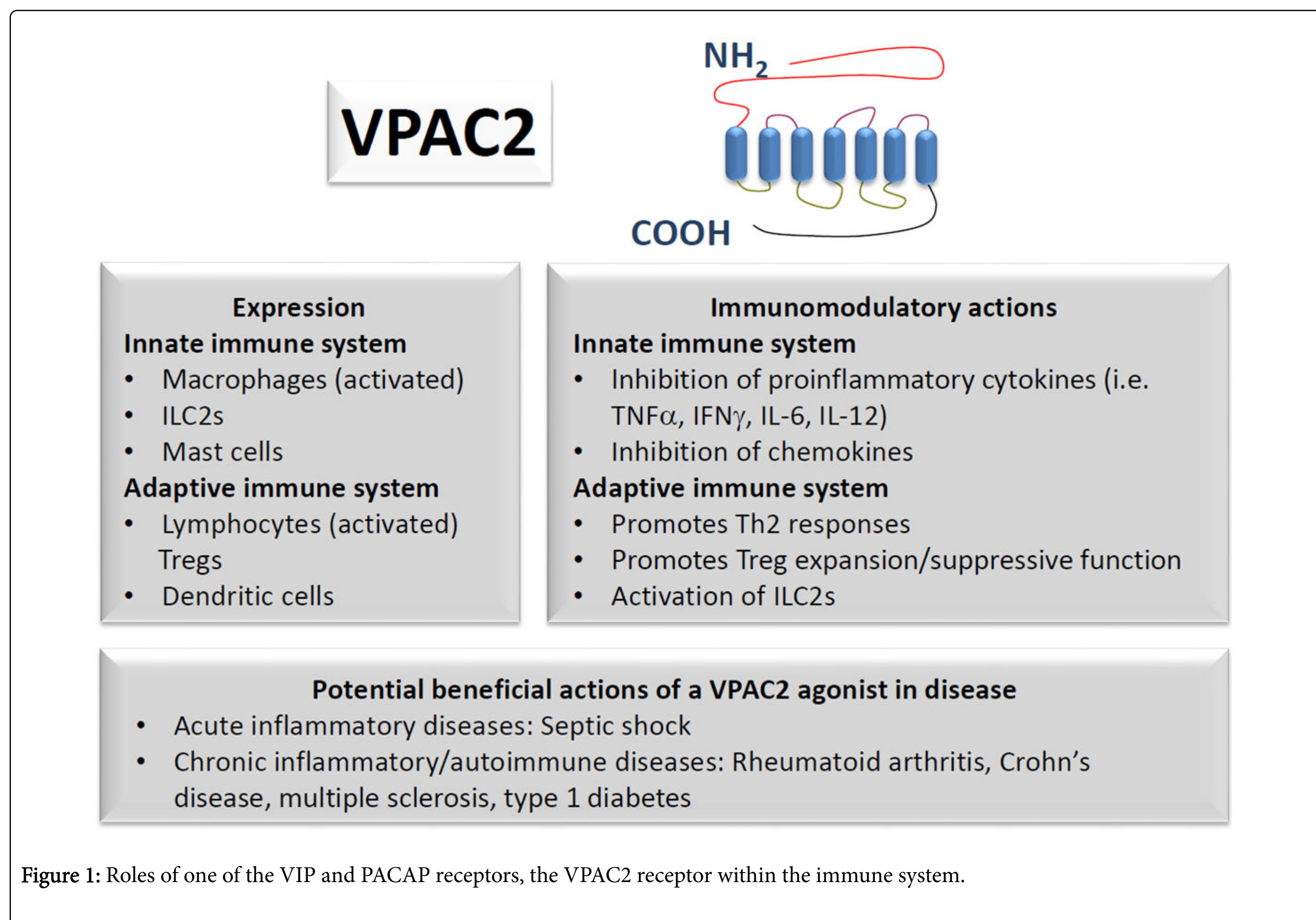

Figure 1: Roles of one of the VIP and PACAP receptors, the VPAC2 receptor within the immune system.

Several VIP analogs with highly specific binding to VPAC2 have been generated. Among them, Ro 25-1392 and Ro 25-1553, are two cyclic derivatives of VIP [18-20]. Although these have been useful to identify VIP-VPAC2 mediated actions in various experimental settings, the presence of N-terminal acylation, cyclization from Lys-21 to Asp-25, C-terminal amidation, and O-Me-Tyr-10 or Nle-17 on these analogs imposes a big challenge for their synthesis, which may hamper their potential use as therapeutic drugs. These difficulties have been bypassed by the generation of a peptidic VPAC2 specific agonist with a simpler structure, BAY 55-9837, which was developed by Tsutsumi et al. through site-directed mutagenesis based on sequence alignments of PACAP, VIP, and related analogs [21]. Substitution of a valine in the aminoacid position 5 by a Cα-methylated valine in Ro 25-1553, and substitution of asparagines in positions 9 and 28 followed by site-specific cysteine conjugation with a 22- or 43-kDa polyethylene glycol (PEG) for BAY 55-9837, have improved the stability of these agonists [22, 23]. Recently, the use of chitosan-decorated selenium nanoparticles (CS-SeNPs) as protein carriers of BAY 55-9837 prolonged its half-life *in vivo* [24]. Due to a potential use of VPAC2 activation for the treatment of diabetes and asthma, most of the efforts have focused on developing VPAC2 agonists. However, a highly specific antagonist for this receptor was generated by myristoylation of the amino-terminus of [K(12)]VIP(1-26) extended carboxyl-terminally with a five aminoacid sequence of Ro 25-1553 [25]. This may be a useful tool in studies to dissect specific roles played by VPAC2.

VPAC2 Expression by Immune Cells

The expression of VIP and PACAP receptors in immune cell types has been described with different patterns. In resting lymphocytes, VPAC1 has been found to be constitutively expressed, and VPAC2 is absent or expressed at very low levels [26-28]. Nevertheless, upon *in vitro* activation of the CD3/TCR complex, VPAC1 is downregulated, at least transiently, and VPAC2 upregulated [26-28]. This has led to the hypothesis that VPAC2 may be the main receptor modulating the functionality of activated T cells, and it may become more relevant than other VIP and PACAP receptors in pathogenic situations that lead to T cell activation. Interestingly, VPAC2 has been reported to be upregulated in T CD4+ cells from HIV patients, which was not associated to the viral load but was suggested to reflect a repetitive exposure to antigens [29]. In another study, a higher VPAC2/VPAC1 ratio was found in memory T helper (Th) cells from early rheumatoid patients compared to healthy subjects, although in this case, this was mostly due to a strong decrease in VPAC1 expression, rather than a strong up regulation of VPAC2 [30].

In addition to wild type (WT) full length VPAC2, splice variants of VPAC2 have been identified by PCR in murine and human lymphocytes [31,32]. In mice lymphocytes, a variant with a 14 aminoacid deletion in the carboxyl-terminal end of the seventh transmembrane domain has been identified, which has the same affinity for VIP as the WT receptor, but does not induce cAMP upon binding [31,32]. In human lymphocytes, a variant with a 114

aminoacid deletion beginning with the carboxyl-terminal end of the third cytoplasmic loop variant was found, with reduced affinity for VIP and multiple functional differences with the WT receptor [32]. Nevertheless, the significance of these variants *in vivo* has not been further investigated.

Some studies have investigated the expression of VPAC2 expression in thymocytes. It has been reported that VPAC2 is the main VIP-PACAP receptor expressed in human thymocytes [33]. However, different results were published in two studies in mice. Delgado et al. found a constitutive expression of VPAC1 and lack of VPAC2, which was induced by PMA and an anti CD3 antibody [27]. On the other hand, Vomhof-DeKrey et al. reported the presence of both VPAC1 and VPAC2 receptors in thymocytes, with a relative expression that varies along maturation: a predominant expression of VPAC1 in earliest thymic progenitor (ETP) and CD4 and CD8 double negative (DN) 1 cells, a switch to VPAC2 in DN2 and DN3 stages, and then back to VPAC1 in DN4 and subsequent double positive (DP) and single positive (SP) stages [34]. Species-specific differences in gene expression may explain the discrepancy between the profile of expression in human and mice thymocytes. In any case, because VPAC2 deficient mice do not exhibit alterations in the frequency of different thymocyte populations, the relevance of VPAC2 expression during thymocyte maturation remains to be elucidated.

In addition to lymphocytes, other immune cell populations have been reported to express VPAC2. For example, it is expressed in peritoneal macrophages and the macrophage cell line Raw 264.7 with a similar pattern to that in lymphocytes: VPAC2 has been reported to be absent or expressed at low levels in unstimulated cells, but is induced upon activation *in vitro* with gram-positive (toll like receptor (TLR)2 ligands) and gram-negative bacteria wall constituents (TLR4 ligands) [35-37]. Moreover, the TLR7 synthetic ligand imiquimod induced VPAC2 mRNA expression. The expression of VPAC2 has been also reported to be low in monocytes from healthy human subjects, but to be elevated in monocytes from patients with Sjogren's syndrome [38]. Other studies, suggest that the inducible nature of VPAC2 expression on macrophages may be tissue specific. For example, a study reported a constitutive expression of VPAC2 in human lung macrophages [39]. Moreover, it has been shown that murine primary microglial cells, which are considered as the resident macrophages of the brain, do not express VPAC2, even after exposure to lipopolysaccharide (LPS), a TLR4 ligand [40]. Other cells, such as murine bone marrow-derived dendritic cells, murine Langerhans cells and human plasmacytoid dendritic cells have been found to express VPAC2 constitutively [41-43]. VPAC2 has been also reported to be expressed on human skin mast cells and in the human mast cell lines HMC1 and LAD2 [44, 45]. In LAD2 cells, VPAC2 became upregulated through IgE/anti-IgE activation.

VIP and PACAP Immunomodulatory Roles

Multiple actions of VIP and PACAP in the immune system have been described [46]. One of the most relevant from a therapeutic standpoint is their ability to inhibit at multiple levels innate and adaptive inflammatory responses [47]. Regarding the innate immune axis, the VIP and PACAP inhibition of chemokine and proinflammatory cytokine production by macrophages has a central role [48,49]. Contributing to its ability to abrogate inflammation, VIP has been shown to down regulate the expression of the pathogen associated molecular pattern receptors of innate immunity toll-like receptors 2 and 4 (TLR2 and TLR4), which was found *in vivo* in tissues undergoing inflammation, but also *in vitro* in isolated peritoneal macrophages and the macrophage cell line Raw 264.7 [50-53]. In addition, VIP and PACAP induce anti-inflammatory mediators such as IL-10 [54]. The actions on a wide variety of inflammatory mediators are possible because downstream of their receptors they modulate several key transduction pathways and factors controlling the expression of a wide range of target genes with immunoregulatory roles (thoroughly reviewed in [55]). This includes the inhibition of one of the most important pathways involved in inflammation, the NF-kB pathway, through inhibition of IkB phosphorylation and subsequent degradation. The involvement of cAMP in this effect is not clear, and seems to vary in different myeloid cell types studied (i.e. peritoneal macrophages, macrophage and monocyte cell lines and microglia), and has been postulated to depend on the cellular differentiation state. Moreover, VIP and PACAP receptors lead to CREB phosphorylation implicated in the synthesis of IL-10 and inhibition of the MAP kinase pathways MEKK1/MEK3/MEK6/p38 and MEKK1/MEK4/JNK, involved in the expression of proinflammatory cytokines. The effects on these pathways contribute to VIP and PACAP down regulation of genes downstream of LPS-TLR4. On the other hand, it was shown that in both macrophages and microglia, VIP blocks IFNγ signal transduction by suppressing Jak1 and Jak2 phosphorylation and therefore STAT1 phosphorylation. This is particularly relevant in chronic inflammation driven by Th1 cells, of which IFNγ, a potent activator of macrophages, is a hallmark cytokine.

Besides their effects on innate immunity, VIP and PACAP modulate adaptive immune responses. In this sense, a well-recognized action for these neuropeptides is their ability to promote Th2 cell differentiation, critically involved in type-2 cell responses. These have a protective role against helminth infection, although in certain circumstances they can be deleterious and lead to chronic allergic diseases. Eosinophils, basophils and mast cells are the ultimate effector cells in these responses, driven by IL-4, IL-5, IL-9 and IL-13 cytokines, all of which are known to be produced by Th2 cells. It has been shown that VIP promotes Th2 polarization *in vitro* and *in vivo* through multiple non-exclusive mechanisms. In this sense, a series of data has shown that VIP modifies the co-stimulatory molecule expression profile and cytokine and chemokine secretion profile in macrophage and dendritic cells, in a manner that promotes Th2 responses [36,56,57]. Moreover, *in vivo*, VIP or PACAP administrations protected certain CD4 Th2 cells from apoptosis and allow the survival of Th2 effectors and the generation of long-lived memory cells [58,59].

The anti-inflammatory properties of VIP and PACAP led to promising therapeutic activities in murine models of acute inflammatory disorders such as septic shock and chronic inflammatory autoimmune diseases such as rheumatoid arthritis, Crohn's disease and multiple sclerosis [49,60-63]. The latter belong to a group of diseases with common pathogenic traits: local inflammation (in different target tissues, such as joints, colon and central nervous system, respectively), driven by autoreactive Th1 cells. Therefore, the fact that VIP and PACAP favor Th2 at expense of Th1 responses played a critical role in their therapeutic effects. In these studies, the peptides were administered intraperitoneally, and the treatments, at doses between 1 to 10 n moles per injection, were most efficient when started at early stages of the disease. VIP and PACAP abrogated the inflammatory response, and switched the T cell phenotype from Th1 to Th2, leading to an amelioration of the clinical symptoms of these diseases.

VPAC2-mediated actions in the immune system

Pharmacological studies with specific receptor agonists have demonstrated that both VPAC1 is the main receptor involved on VIP and PACAP anti-inflammatory actions *in vitro* and *in vivo*, and VPAC2 has a partial role [49,60-62,64]. Likewise, VIP and PACAP effects on Th2 polarization through accessory cells have been suggested to be largely dependent on VPAC1. Nevertheless, other studies have involved VPAC2 in the ability of VIP to induce Th2 responses directly on T cells. In this sense, lymphocytes from mice that were genetically modified to express human VPAC2 constitutively in T CD4+ cells (VPAC2 TG) exhibit a Th2 phenotype with production of IL-4 and IL-5 in response to TCR stimulation [65]. These mice were found to be naturally in an allergic state with elevated IgE, IgG1 and eosinophils in blood. Moreover, these mice exhibited reduced hapten-induced delayed-type hyper sensibility (DTH), due to a higher Th1/Th2 ratio. In a complementary study, the same team showed that mice with a global deletion of VPAC2 (knock-out mice (KO)) [12], developed the opposite phenotype. Although different from the VPAC2 TG mice, VPAC2 KO seem normal in a resting state, as expected, these mice exhibited enhanced DTH in response to hapten [66]. Conversely, these mice developed reduced immediate-type hyper sensibility allergic responses to hapten, with diminished blood IgE levels and cutaneous anaphilactic responses. Regarding the mechanisms of action by which VIP/VPAC2 induce the cells to become Th2, it was found that this is mediated by an up regulation of certain Th2-related transcription factors (i.e. c-Maf and JunB, but not GATA-3), which consequently enhanced IL-4 and IL-5 production [67].

The fact that DTH is exacerbated in VPAC2 KO mice, with enhanced Th1 vs. Th2 cytokines, suggest that this receptor may play a protective role against Th1-driven diseases. Recently, we subjected VPAC2 KO mice to experimental autoimmune encephalomyelitis (EAE), a model of multiple sclerosis (MS) [68]. MS is a chronic inflammatory disease of the CNS thought to be driven by auto reactive T cells against myelin peptides. A role of Th1 and Th17 cells in the pathogenesis of the disease has been implied by multiple studies. We found that VPAC2 KO mice exhibited exacerbated clinical EAE, with increased immune cell infiltration and demyelination compared to WT mice. This phenotype was associated to elevated Th1 and Th7 cell but reduced Th2 responses. In addition, these mice exhibited a striking deficiency in the number of regulatory T cells (Tregs) (identified as CD4+CD25+Foxp3+) in the CNS, lymph nodes and thymus. The latter has been suggested to be the main source for Tregs during EAE [69]. This is in agreement with multiple publications supporting that VIP promotes Treg generation and functionality (reviewed in [70]). A similar response to EAE induction was found in PACAP KO mice [71,72], implying that VPAC2 mediates the anti-inflammatory actions of PACAP. PACAP/VPAC2 signaling could contribute to the maintenance and expansion of Tregs directly, as we found that VPAC2 is expressed in these cells. Supporting this possibility, we found that the expansion of Tregs isolated from VPAC2 KO mice with anti-CD3/CD28 beads and IL-2 *in vitro* was diminished compared to that of WT Tregs. Moreover, the *in vitro* suggests that PACAP/VPAC2 pathway is critical to maintain normal Treg expansion and activity.

In another study, Yadav et al. [73] demonstrated that VPAC2 KO mice exhibited greater weight loss and intestinal histopathology than WT mice in the model of dextran sodium sulfate (DSS)-induced colitis. Probably contributing to this exacerbated response, the levels of certain proinflammatory mediators (IL-6, IL-1β and MMP-9) were higher in VPAC2 KO vs. WT mice. This phenotype could be related to an immunosuppressive role for this receptor. Nevertheless, the authors suggested that it might reflect proinflammatory actions of a VIP/VPAC1 signaling axis, which could be responsible for the reduced DSS-pathology they observed in VPAC1 KO mice. In fact, the same team previously demonstrated that VIP through VPAC1 lead to the differentiation of T lymphocytes into Th17 cells [74]. The fact that VIP could promote immune responses is also supported by previous data reporting stimulatory actions on IL-6 secretion and a role in chemo taxis. Nevertheless, further investigations are required to dissect the dual roles of VIP in immunity.

In addition to the cell types mentioned above, group 2 innate lymphoid cells (ILC2) have been more recently identified as effectors of type 2 responses [75]. ILCs are a population of lineage-negative (Lin−, i.e. lacking surface markers for T, B, NK and monocytes/macrophage lineages) lymphocyte-like cells which offer critical first-line immune responses against pathogens. Despite their lack of T cell and B cell antigen receptors (TCRs and BCRs), they can produce effector cytokines comparable to those produced by CD4+ Th cell subsets. Among all ILCs, ILC2 cells were initially identified in mice as MHC class IIhigh, CD11null and Lin− cells, which amplified type 2 immune responses upon treatment with IL-25, a Th2-produced cytokine [76]. In mice, the existence of two ILC2 populations, natural (nILC2) vs. inflammatory (iILC2), has been proposed [77]. These are different in that whereas the former can be found in homeostatic conditions and respond to IL-33, the latter rapidly expands in response to N. brasiliensis infection or IL-25 but not IL-33 administration. ILC2 have been later identified in several locations in humans, including lung, intestine and skin [78-81]. Recently, VIP has been suggested to modulate the activity of ILC2s through its VPAC2 receptor [82]. In this study, it was found that VPAC1 and VPAC2 are expressed in both gut and lung ILC2s. In addition, it was shown that VIP, as well as a VPAC2 agonist stimulated at similar levels the production of IL-5 by isolated intestinal Lin−CD45+KLRG1+ ILC2 cells in the presence of IL-7. Because VIP-innervation is rich in the intestine and lung, where these cells are abundant, this could be a natural regulator factor of ILC2s. In addition, it has been shown by using TCR transgenic mice that Th2 cells produce VIP, suggesting that in fact this neuropeptide could act as a Th2 cytokine, and suggesting a potential new mechanism by which Th2 cells may modulate the activity of ILC2s [83].

VPAC2 perspectives as a therapeutic target

Although there are very few studies investigating the effects of VIP or PACAP administration to patients with inflammatory pathologies, these have provided encouraging results. For example, the effect of synthetic VIP (aviptadil) administration by inhalation to patients with sarcoidosis, an inflammatory Th1-driven systemic disease characterized by granuloma formation mainly in the lung, has been investigated. In this study, the patients exhibited an amelioration of the symptoms, with no adverse side effects [84]. This effect was associated with a reduction in the levels of TNFα but an increase of Tregs in bronchoalveolar lavage. In another study, inhaled aviptadil ameliorated pulmonary hypertension due to its action as a vasodilator [85]. Promising preclinical studies by the team of Pr. Gomariz have tested the actions of VIP in cultures of synovial fibroblasts and peripheral T cells from rheumatoid arthritis patients, and support the anti-inflammatory potential of this peptide [30,53,86-89]. *In vitro* studies using samples from patients suffering inflammatory human diseases (as it has been done for arthritis), or animal models of disease, seem to

be the most suitable approaches to dissect which VIP/PACAP receptor mediates their beneficial actions, and should precede clinical trials.

Studies comparing the outcomes of an administration of VIP and PACAP receptor agonists in the murine models of LPS-induced endotoxemia, collagen-induced arthritis and multiple sclerosis have shown a superior efficacy of VPAC1 vs. VPAC2 and PAC1 agonists. Nevertheless, the fact that VPAC2 KO mice exhibited exacerbated EAE, strongly support the antiinflammatory potential of this receptor. Thus, perhaps the idea of targeting VPAC2 for the treatment of those diseases in humans should not be completely abandoned. In fact, different factors such as the time (i.e. of highest upregulation in immune cells), route or length of administration of a VPAC2 agonist may improve its beneficial effects. Moreover, targeting VPAC2 may contribute to reduce potential VIP or PACAP secondary effects driven by VPAC1. In this sense, in a study testing the efficiency of a VPAC2 agonist for the treatment for asthma based on the bronchodilatory effects of VIP, Ro 25-1553 given by inhalation did not cause adverse effects, at least at short term [90]. Finally, although the studies in mice support the use of a VPAC1 vs. VPAC2 agonist for inflammatory disorders, this remains to be proven in humans, where due to differences in receptor expression among species or in the mechanisms implicated in human disorders vs. their animal models, this may not be true. Future studies will be required to further approach the use of VPAC2 in the clinic.

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