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A NOVEL VIP SIGNALING PATHWAY IN T CELLS cAMP→ Protein Tyrosine Phosphatase (SHP-2?)→JAK2/STAT4→Th1 differentiation

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Abstract

Vasoactive intestinal peptide (VIP) is a potent anti-inflammatory agent. In addition to the deactivation of macrophages, dendritic cells, and microglia, VIP shifts the Th1/Th2 balance, promoting the preferential differentiation and survival of Th2 cells, to the detriment of the proinflammatory Th1 effectors. Several mechanisms operate in the Th1/Th2 shift induced by VIP. Here we report on a novel mechanism for the effect of VIP on T cell differentiation, and show that VIP inhibits Th1 differentiation by interfering directly with the IL-12 Jak2/STAT-4 signaling pathway in T cells. The effect of VIP is cAMP-dependent, and appears to be mediated through the activation of protein tyrosine phosphatases (PTP), with SHP-2 as a potential target. The activation of PTPs represents a novel cAMP-downstream target for the immunomodulatory effects of VIP.

Keywords

Vasoactive intestinal peptide; Th1 differentiation; Jak2/STAT-4 signaling pathway; Protein Tyrosine Phosphatases (PTP); SHP-2 protein tyrosine phosphatase

1. Introduction

Vasoactive intestinal peptide (VIP) is a potent immunosuppressive agent, affecting both innate and adaptive immunity [11-14,16]. Aside from its anti-inflammatory effects on innate immune cells such as macrophages, dendritic cells, and microglia, VIP also affects T cell differentiation and function.

Several authors reported on the role of VIP in promoting Th2-type responses in vivo and in vitro [6,7,9,14,15,17,20,31,32]. A number of different mechanisms are involved in the VIP-mediated shift in the Th1/Th2 balance. On one hand, VIP affects antigen-presenting cells such as macrophages and dendritic cells, inhibiting IL-12 release and promoting CD86 expression [4,7-9]. The decrease in IL-12 inhibits Th1 differentiation, and the VIP-induced increase in CD86 has been associated with its Th2 promoting effect [4,9]. On the other hand, VIP affects

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T cells directly. VIP was shown to promote Th2 and inhibit Th1 differentiation in the absence of antigen-presenting cells [14], to upregulate the expression of the Th2-specific transcriptional factors c-Maf and JunB [30], and to promote Th2 survival by inhibiting FasL and granzyme B expression in Th2, but not Th1 effectors [28]. In addition, VIP was shown to promote the specific attraction of Th2 effectors through the upregulation of Th2-chemoattractants such as CCL22, and the inhibition of Th1-chemoattractants such as CXCL10 [5].

Following activation through the T cell receptor and signaling through CD28, naïve T cells differentiate into three subsets of effector T cells, i.e. Th1, Th2, and the recently identified Th17 cells [21,27,34]. Differentiation into effectors depends on several factors present in the microenvironment, with cytokines as major players. Members of the IL-12 family, primarily IL-12p70 and IL-27 promote Th1 differentiation, whereas IL-4 is a required cytokine for Th2 development, and TGF β , IL-6, and IL-23 are essential for Th17 differentiation. Since IL-12 plays such an important role in the generation of Th1 cells, it was surprising that VIP could shift the Th1/Th2 balance in T cells cultures even in Th1 polarizing conditions, i.e. in the presence of IL-12 [14]. This finding suggested that VIP might interfere with the JAK2/TYK2 $\rightarrow$ STAT4 signaling pathway involved in the IL-12p70-induced Th1 differentiation [23,33]. Here we investigate the effects of VIP on JAK2/STAT4 phosphorylation and on the activation of SHP-2, a phosphotyrosine phosphatase (PTP) involved in the control of cytokine receptor signaling.

2. Experimental/Materials and Methods

2.1. Mice

Male B10.A mice (males; 6-8 weeks old) were purchased from Jackson Laboratories (Bar Harbor, ME) and were maintained in the Rutgers University (Newark, NJ) and Temple University School of Medicine (Philadelphia, PA) animal facilities under pathogen free conditions.

2.2. Reagents

Medium: RPMI 1640 (Gibco Research Lab, Grand Island, NY) containing 10% heat-inactivated FBS (Atlanta Biologicals, Norcross, GA), 2mM L-glutamine (Gibco), 2 mM antibiotics (Gibco) and 50 μ M B-mercaptoethanol (Sigma). Recombinant murine IL-12, IL-2 and IFN γ were purchased from Peprotech (Rock Hill, NJ); CD4 (L3T4) MicroBeads was obtained from Miltenyi Biotec (Auburn, CA); purified hamster anti-mouse CD3, anti-mouse CD28, purified and biotinylated rat anti-mouse IFN γ antibody, GolgiPlug and Cytotfix/Cytoperm kits were purchased from BD Biosciences/PharMingen (San Diego, CA); streptavidin-peroxidase, PMA and ionomycin were purchased from Sigma (St. Louis, MO); TMB Elisa substrate was purchased from Pierce Chemical Co. (Rockford, IL); VIP, dibutylcAMP (dbcAMP), forskolin, H89, bpV (phen), sodium orthovanadate, okadaic acid (OA) and the PKA inhibitor KT5720 were obtained from Calbiochem (La Jolla, CA). The following antibodies were used for FACS analysis: PE-conjugated rat anti-mouse IFN γ and PE-conjugated rat IgG1 (PharMingen), rabbit IgG (Sigma), rabbit anti-STAT4 and rabbit anti-phosphorylated (Y693)-STAT4 (Zymed), phospho-Jak2 (Tyr 1007/1008) antibody, rabbit polyclonal anti-mouse SHP-2, rabbit polyclonal anti-mouse phosphorylated SHP-2 (Cell Signaling) and FITC-conjugated goat anti-rabbit IgG (Sigma).

2.3. Isolation and purification of splenic naïve CD4 $^{+}$ T cells from spleen

CD4 $^{+}$ T cells were purified from B10.A spleen by immunomagnetic separation using anti-CD4 coupled magnetic microbeads and the autoMACS system (Miltenyi Biotec, Bergish-Gladbach, Germany). The purity of the isolated cells was determined by FACS analysis (>98% for CD4 $^{+}$ cells).

2.4. Cell culture and ELISA assays

Purified CD4⁺ T cells were cultured in 48-well plates (2×10^6 cells/ml; final volume 0.5ml) and activated with immobilized anti-CD3 (3 μ g/ml) and anti-CD28 (1 μ g/ml) in the presence of rmIL-2 (10ng/ml) and rmIL-12 (10ng/ml) for 5 days. On days 0, 2 and 4, VIP (10^{-6} - 10^{-10} M) was added. On day 5, the cells were restimulated with immobilized anti-CD3 (3 μ g/ml) and anti-CD28 (3 μ g/ml) in the presence of IL-2 (10ng/ml) for 48 hr. Cell-free supernatants were harvested and subjected to IFN γ ELISA. For the ELISA assay we used purified anti-IFN γ polyclonal antibody as capture Ab (2 μ g/ml), followed by detection with a biotinylated anti-mouse IFN γ antibody (1 μ g/ml). The sensitivity of the ELISA assay is 20pg /ml IFN γ .

2.5. FACS analysis for intracellular IFN γ

Purified CD4⁺ T cells were cultured as for the ELISA assay. On day 5, the cells were restimulated for 4hr with PMA (5 ng/ml) and ionomycin (500ng/ml) in the presence of protein transport inhibitor GolgiPlug. The cells were fixed and permeabilized with the Cytotfix/Cytoperm Kit (BD Biosciences/ PharMingen) according to the manufacturer's instructions. Cells were stained with PE-conjugated rat anti-mouse IFN γ for 30 min and analyzed by FACS. PE-conjugated rat IgG1 was used as a control.

2.6. FACS analysis for p-STAT, p-JAK, and p-SHP-2

Purified CD4⁺ T cells were cultured in 24-well plates (2×10^6 cells/ml) precoated with immobilized anti-CD3 (3 μ g/ml) and anti-CD28 (3 μ g/ml), in the presence of IL-2 (10ng/ml) for 48hr. The cells were washed and allowed to rest for 24 hr in serum-free media. After two washes, the cells were resuspended in PBS with 2% FBS at a concentration of 3×10^6 cells/ml. 500 μ l aliquots were incubated with IL12 (100ng/ml), VIP (10^{-6} - 10^{-10} M), dbcAMP (10^{-4} M), forskolin (10^{-5} M) or H89 (10^{-6} M) at 37° C for the indicated time periods. Cells treated with PBS were used as controls. Following incubation, the cells were fixed and permeabilized with Cytotfix/Cytoperm according to the manufacturer's instructions. The cells were incubated with various antibodies: rabbit polyclonal anti-phosphorylated STAT4 (pSTAT4), rabbit polyclonal anti-STAT4 antibody, phospho-Jak2 (Tyr 1007/1008), anti-SHP-2, anti-phosphorylated SHP-2, and rabbit IgG at room temperature for 30min, followed by the appropriate FITC-conjugated goat anti-rabbit IgG for 30min. After extensive washing, the cells were analyzed by FACS.

3. Results

3.1 VIP inhibits IL-12-induced Th1 responses

Naïve CD4⁺ T cells undergo differentiation into Th1 and Th2 effectors upon antigenic stimulation. Differentiation into Th1 effectors is mediated primarily by IL-12 secreted by stimulated dendritic cells or macrophages. VIP has been reported to promote Th2 differentiation, while reducing Th1 responses both in vivo and in vitro. To determine whether VIP interferes with IL-12 signaling in differentiating CD4⁺ T cells, we stimulated purified naïve CD4⁺ T cells for 5 days with immobilized anti-CD3 and anti-CD28 Abs in the presence of IL-2 plus the Th1 polarizing cytokine IL-12. On days 0, 2, and 4 various concentrations of VIP (10^{-6} - 10^{-10} M) were added to the cultures. On day 5, the cells were replated and restimulated with immobilized anti-CD3 and anti-CD28 in the presence of IL-2 for 48 hr. Since IFN γ is the major Th1-type cytokine, cell-free supernatants were harvested and subjected to IFN γ Elisa assays. Whereas unstimulated T cells did not secrete IFN γ , T cells activated in the presence of IL-12 produced significant levels of IFN γ . The presence of VIP during the differentiation step resulted in a dose-dependent decrease in IFN γ release (Fig. 1A).

To investigate whether the inhibition of IFN γ production by VIP was the direct consequence of a reduction in the number of IFN γ -secreting cells, we determined the number of intracellular IFN γ positive cells by FACS. Naïve CD4 $^{+}$ T cells were stimulated with immobilized anti-CD3 and anti-CD28 in the presence of IL-2 plus IL-12 for 5 days. VIP (10^{-6} , 10^{-8} and 10^{-10} M) was added on days 0, 2 and 4. The cells were replated and restimulated with PMA and ionomycin, and subjected to FACS analysis for intracellular IFN γ . VIP reduces the number of IFN γ -secreting cells in a dose-dependent manner, without affecting the fluorescence intensity per cell (Fig. 1B).

3.2 VIP inhibits IL-12-induced tyrosine phosphorylation of STAT4 in CD4 $^{+}$ T cells

IL-12 signaling involves the Jak/STAT pathway, inducing Jak2/Tyk2 and STAT4 phosphorylation. To determine whether VIP affects IL-12 signaling, we first assessed its capacity to affect STAT-4 phosphorylation. Following stimulation of CD4 $^{+}$ T cells with immobilized anti-CD3 and anti-CD28 Abs in the presence of IL-2 for 48h, the cells were washed, and restimulated with anti-CD3 plus anti-CD28 in serum-free conditions for 24h, followed by IL-12 treatment in the presence or absence of VIP. The initial stimulation is necessary for the cells to start expressing functional IL-12R, and the restimulation in the absence of serum is required to avoid a high background in STAT-4 phosphorylation. The levels of STAT-4 and phosphorylated STAT-4 were determined by FACS. Time-course experiments (10, 20, 30, and 60 min) established that maximum STAT-4 phosphorylation occurred 10 min following IL-12 treatment. Pretreatment of T cells with VIP (10^{-7} M) for 30 min prior to IL-12 addition inhibited STAT-4 phosphorylation without affecting total STAT-4 levels (Fig. 2A). The effect of VIP was dose-dependent with significant inhibitory effects in the 10^{-6} to 10^{-9} M range (Fig. 2B). Pretreatment of cells with VIP for 10 min followed by IL-12 inhibited STAT-4 phosphorylation to the same degree as the simultaneous addition of VIP and IL-12 (Fig. 2C). In contrast, addition of VIP 5 min after IL-12 did not inhibit STAT-4 phosphorylation (Fig. 2C). These results indicate that the VIP-induced signals have to be present when IL-12 signaling is initiated to exert an inhibitory effect.

3.3. VIP inhibits IL-12 induced JAK2 phosphorylation

Since IL-12 induced Jak2/Tyk2 phosphorylation precedes the phosphorylation of STAT-4, we investigated the effect of VIP on IL-12-induced JAK2 phosphorylation. As for the STAT-4 experiments, CD4 $^{+}$ T cells were stimulated with immobilized anti-CD3 and anti-CD28 in the presence of IL-2 for 48h, washed, and restimulated with anti-CD3 plus anti-CD28 in serum-free conditions for 24h, followed by IL-12 treatment in the presence or absence of VIP. Jak-2 phosphorylation was determined by FACS 5 min after the addition of IL-12. Similar to STAT-4, VIP reduced phosphorylated JAK2 levels in a dose-dependent manner, without affecting JAK2 levels (Fig. 3A and B). These results indicate that VIP inhibits IL-12-induced JAK2 phosphorylation in CD4 $^{+}$ T cells.

3.4. VIP inhibits IL-12-induced STAT-4 phosphorylation through the cAMP-PKA signaling pathway

The effects of VIP in T cells are mediated through VPAC1 and VPAC2 receptors, which signal primarily through the activation of adenylate cyclase. To confirm the involvement of cAMP in the effects of VIP on STAT-4 phosphorylation, we treated CD4 $^{+}$ T cells with dbcAMP or forskolin prior to IL-12 addition. Both agents mimicked VIP in terms of inhibition of STAT-4 phosphorylation (Fig. 4A). In addition, H89, a PKA inhibitor, reversed the inhibitory effect of VIP (Fig. 4B). These results indicate that the inhibition of the IL-12 signaling pathway by VIP is mediated through the cAMP-PKA pathway.

3.5. The inhibitory effect of VIP on STAT-4 phosphorylation is prevented by protein tyrosine phosphatase (PTP) inhibitors

We next investigated whether the inhibitory effect of VIP on IL-12-induced STAT-4 phosphorylation might involve the activation of a PTP. CD4⁺ T cells were pretreated for 15 min with 100 μ M bpV (phen) or 1mM sodium orthovanadate (Na_3VO_4) which inhibit PTPs, or with 50 nM okadaic acid (OA) which inhibits protein serine-threonine phosphatase 1 and 2A, but not PTPs, followed by exposure to VIP for 30 min, and stimulation with IL-12. Both bpV (phen) and sodium orthovanadate, but not by okadaic acid, prevented the inhibitory effect of VIP (Fig. 5), suggesting that a phosphatase belonging to the family of protein tyrosine phosphatases mediates the effect of VIP on the IL-12 signaling pathway.

3.6 VIP induces phosphorylation of the PTP SHP-2

Since SHP-2, one of the PTP involved in cytokine signaling, has been reported to be activated by increases in cAMP [25,37], we assessed whether VIP induced SHP-2 phosphorylation in T cells exposed to IL-12. CD4⁺ T cells were treated with IL-12 in the presence or absence of VIP (10^{-7} M) and the levels of phosphorylated SHP-2 were determined by flow cytometry. VIP induced SHP-2 phosphorylation (Fig. 6). In a time course experiment, the VIP-induced SHP-2 phosphorylation in a dose-dependent manner, without affecting total SHP-2 levels (Figs. 6 and 7).

3.7 VIP-induced SHP-2 phosphorylation is PKA-dependent

Since the major signaling pathway in T cells treated with VIP is the cAMP $\rightarrow$ PKA pathway, we tested the effect of two PKA inhibitors, H89 and KT5720 on VIP-induced SHP-2 phosphorylation. CD4⁺ T cells were preincubated for 30 min with either H89 or KT5720, followed by treatment with VIP. H89 and KT5720 alone did not affect cell viability or SHP-2 phosphorylation. Both inhibitors reduced VIP-induced SHP-2 phosphorylation to control levels (medium) (Fig. 8), which indicating that the effect of VIP on SHP-2 phosphorylation is mediated through PKA. This is similar to the effect of VIP on STAT-4 phosphorylation (Fig. 4B).

4. Discussion

VIP is a potent anti-inflammatory agent, affecting both innate and adaptive immunity. In addition to the deactivation of inflammatory cells such as macrophages, dendritic cells, and microglia, VIP shifts the Th1/Th2 balance in favor of Th2-responses [6,7,9,14,15,17,31,32]. The preferential induction of Th2 responses by VIP is mediated through several molecular mechanisms. On one hand, VIP affects the Th1/Th2 balance through effects on APCs, such as inhibition of IL-12 production which impacts negatively Th1 differentiation, and upregulation of CD86 which supports Th2 differentiation [4,7-9]. On the other hand, VIP affects T cells directly by upregulating the Th2 master transcriptional factors c-Maf and JunB, and by promoting the preferential survival of Th2 effectors and memory cells [6,28,30]. In addition, VIP also induces the preferential accumulation of Th2 effectors by promoting the release of Th2-specific chemoattractants [5].

Although the VIP-induced upregulation of the Th2 master transcriptional factors c-Maf and JunB is responsible for the positive effect of VIP on Th2 differentiation, the inhibition of Th1 differentiation even in the presence of the polarizing cytokine IL-12p70 remains unexplained. In the present study we offer evidence that VIP, administered before or at the same time with IL-12, inhibits IL-12R-induced Jak2/STAT-4 phosphorylation. The VIP effect on Jak2/STAT-4 activation is mediated through the cAMP $\rightarrow$ PKA pathway, and is prevented by protein tyrosine phosphatase (PTP) inhibitors. VIP also induces the phosphorylation and activation of the PTP SHP-2 through the cAMP $\rightarrow$ PKA pathway. We propose that the VIP-induced

activation of SHP-2 results in dephosphorylation of Jak2/STAT-4, silencing of the IL-12R signaling pathway, and subsequent inhibition of Th1 differentiation.

Most of the anti-inflammatory effects of VIP in macrophages/microglia are mediated through the inhibition of NF κ B and MAPK signaling pathways [12,13]. With the exception of one previous publication from our laboratory [3], there are no reports on the VIP effect on Jak2/STATs-mediated signaling initiated by cytokine receptors. We reported previously that VIP affects IFN γ -induced Jak1/STAT-1 signaling in macrophages through a cAMP-PKA-dependent pathway [3]. However, the mediator involved in the dephosphorylation of Jak1/STAT-1 remained unidentified. Based on the results reported here for Jak2/STAT-4 signaling, it is possible that PTPs, particularly SHP-2, could be also involved in the VIP-induced dephosphorylation of the Jak1/STAT-1 signaling pathway.

Numerous PTPs have been identified and characterized in immune cells (reviewed in [10, 22]). Among those, SHP-2 was reported to be phosphorylated and activated through the cAMP $\rightarrow$ PKA pathway both in T cells and adrenocortical cells [25,37]. Although SHP-2 was traditionally considered a positive regulator in immune cells, recent reports indicate that SHP-2 plays also a negative regulatory role in immune cell activation [2,29,35,36]. Particularly relevant to IL-12 signaling and Th1 differentiation, SHP-2 was reported to directly associate and dephosphorylate Jak2/Tyk2 [1,18,19,24,26]. These findings made SHP-2 an attractive putative target for the inhibitory effect of VIP on the Jak2/STAT-4 signaling pathway. We found indeed that VIP induces SHP-2 phosphorylation in primary T cells exposed to IL-12, and that the VIP-induced SHP-2 phosphorylation is PKA-dependent. In addition, the VIP-induced dephosphorylation of Jak2/STAT-4 could be reversed by PTP inhibitors. Based on the results presented in this report, we propose that VIP, present during Th1 differentiation blocks IL-12 signaling through the activation of the PTP SHP-2 (Fig. 9). However, although SHP-2 is an attractive target for the VIP effect on the IL-12 signaling pathway and subsequent Th1 differentiation, a direct connection has to be established through use of dominant negative, phosphorylation-deficient SHP-2.

5. Conclusions

VIP acts as a potent anti-inflammatory agent, by deactivating innate immune cells such as macrophages, dendritic cells, and microglia, and by shifting the T cell response from a proinflammatory Th1-type, to an anti-inflammatory Th2-type response. The shift in the Th1/Th2 balance is mediated through different mechanisms, including the direct inhibition of T cell differentiation into Th1 effectors. Here we present a novel mechanism for the VIP effect on Th1 differentiation, i.e. the negative interference with the IL-12 signaling pathway in differentiating T cells. We demonstrate that the VIP treatment of IL-12 stimulated CD4 $^{+}$ T cells results in the dephosphorylation of Jak/STAT-4, through a cAMP $\rightarrow$ PKA-dependent mechanism. This process can be reversed in the presence of protein tyrosine phosphatase (PTP) inhibitors. We also show that VIP induces the phosphorylation/activation of the PTP SHP-2 in differentiating T cells, in a PKA-dependent manner. Based on these results, we propose that VIP activates SHP-2 through the cAMP $\rightarrow$ PKA signaling pathway, and that the activated phosphatase dephosphorylates Jak2, turning off the IL-12 Jak2/STAT-4 signaling pathway and preventing T cell differentiation into Th1 effectors. Although SHP-2 represents an attractive target for the VIP-induced inhibition of Jak2/STAT-4 signaling, a direct connection remains to be established. However, regardless of the exact nature of the PTP, the fact that VIP affects IL-12 signaling directly in differentiating T cells, by inhibiting Jak2/STAT-4 phosphorylation in a cAMP-dependent manner, and that this event is mediated through the activation of a protein tyrosine phosphatase, represents a novel mechanism for the potent anti-inflammatory effect of VIP, defining a new base for the potential use of this neuropeptide as a therapeutic agent.

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Fig. 1. VIP inhibits the IL-12-induced Th1 response

Naïve CD4⁺ T cells (2×10^6 cells/ml) were stimulated with immobilized anti-CD3 ($3 \mu\text{g/ml}$) and anti-CD28 ($1 \mu\text{g/ml}$) Abs in the presence of IL-2 (10 ng/ml) and IL-12 (10 ng/ml) for 5 days. On days 0, 2 and 4, VIP was added (10^{-6} - 10^{-10} M). On day 5, the cells were replated and restimulated with immobilized anti-CD3 ($3 \mu\text{g/ml}$) and anti-CD28 ($3 \mu\text{g/ml}$) in the presence of IL-2 for 48 hr. IFN γ released in the supernatant was measured by ELISA (A). * indicates statistically significant differences compared to the anti-CD3 and anti-CD28 activated control (no VIP); $p < 0.01$. Additional controls consist of unstimulated T cells cultured with medium or with 10^{-7} M VIP. Cells treated as above were replated, restimulated with PMA (5 ng/ml) and ionomycin (500 ng/ml) for 4 hr in the presence of the protein transport inhibitor GolgiPlug and subjected to FACS analysis (B). One representative experiments out of five is shown.

Fig. 2. VIP inhibits IL-12-induced STAT4 tyrosine phosphorylation

(A) CD4⁺ T cells were stimulated with immobilized anti-CD3 and anti-CD28 plus IL-2 for 48 hr. After extensive washing, the cells were replated in serum-free medium for 24hr, treated with VIP for 30 min, followed by IL-12 (100ng/ml) for 10 min. The cells were permeabilized and stained for P-STAT4 and total STAT4. (B). Cells treated as in (A) were preincubated with different concentration of VIP (10^{-6} - 10^{-10} M) for 30 min, followed by IL-12 (100ng/ml) for 10 min. (C). CD4⁺ T cells were treated as in (A). VIP (10^{-7} M) was added at different times: 10 min before IL-12, same time as IL-12, 5 min after IL-12. One experiment out of three is shown.

Fig. 3. VIP inhibits IL-12-induced JAK2 tyrosine phosphorylation

CD4⁺ T cells were stimulated with immobilized anti-CD3 and anti-CD28 plus IL-2 for 48 hr. After extensive washing, the cells were replated in serum-free medium for 24hr, treated with VIP (10^{-7} M) (A) or different concentrations (B) for 30 min, followed by IL-12 (100ng/ml) for 5 min. After permeabilization, the cells were subjected to FACS analysis for intracellular P-JAK2 and total JAK2.

Fig. 4. The effect of VIP on STAT4 phosphorylation is mediated through the cAMP-PKA pathway (A). Cells were cultured as in Fig. 2A. Following replating in serum-free conditions for 24h, the cultures were treated with VIP (10^{-7} M), dbcAMP (10^{-4} M) or forskolin (10^{-5} M) for 30 min, followed by IL-12 for 10 min and intracellular staining for P-STAT4. **(B).** Cultures were pretreated with H89 (10^{-6} M) for 15 min, followed by VIP (10^{-7} M) for 30 min and IL-12 for 10 min. The cells were stained for intracellular P-STAT4. One representative experiment out of three is shown.

Fig. 5. PTP inhibitors prevent the inhibitory effect of VIP on IL-12-induced STAT4 phosphorylation

(A). Cells were cultured as in Fig. 2A. Following replating in serum-free conditions for 24h, the cultures were preincubated with bpV (phen) (100 μ M) (A), Na₃VO₄ (1mM) (B), or okadaic acid (OA, 50 nM) (C) for 15 min, followed by VIP (10^{-7} M) for 30 min and IL-12 for 10 min. The permeabilized cells were stained for intracellular p-STAT4. Pnc experiment out of four is shown.

Fig. 6. VIP induces SHP-2 phosphorylation in IL-12-treated CD4⁺ T cells

CD4⁺ T cells were stimulated with immobilized anti-CD3 and anti-CD28 plus IL-2 for 48 hr. After extensive washing, the cells were replated in serum-free medium for 24hr, treated with VIP (10^{-7} M) and IL-12 (100ng/ml) for 20 min. The cells were permeabilized and stained for P-SHP-2. Med- medium control. One experiment out of three is shown.

Fig. 7. VIP induces SHP-2 phosphorylation without affecting total SHP-2 levels

Cells were treated as in Fig. 6. After replating in serum-free medium for 24hr, cultures were treated with different concentrations of VIP for 20 min, permeabilized and stained for P-SHP-2 and total SHP-2. Med - medium control. One experiment out of three is shown.

Fig. 8. VIP-induced SHP-2 phosphorylation is mediated by PKA

Cells were treated as in Fig. 6. After replating in serum-free medium for 24hr, cultures were pretreated with the specific PKA inhibitors H89 (A) or KT5720 (B) for 15 min, followed by VIP (10^{-7} M) for 20 min, permeabilized and stained for P-SHP-2. Med-medium control. One experiment out of three is shown.

Fig. 9. Model for the inhibitory effect of VIP on IL-12 signaling

VIP acting through the VPAC1/2/ receptors activates adenylate cyclase resulting in the activation of PKA. PKA phosphorylates SHP-2, a protein tyrosine phosphatase which in turn acts on Jak2 and possibly directly on STAT4. Dephosphorylation of Jak2 and STAT4 interrupts the IL-12 signaling pathway and prevents the expression of genes involved in Th1 differentiation and function.