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Differential Expression of Vasoactive Intestinal Peptide and Its Functional Receptors in Human Osteoarthritic and Rheumatoid Synovial Fibroblasts

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Objective. **Vasoactive intestinal peptide (VIP) has shown potent antiinflammatory effects in murine arthritis and ex vivo in human rheumatoid arthritis (RA) synovial cells. To investigate the potential endogenous participation of this system in the pathogenesis of RA, we analyzed the expression and regulation of VIP and its functional receptors in human fibroblast-like synoviocytes (FLS) from patients with osteoarthritis (OA) and patients with RA.**

Methods. **The expression of VIP was studied by reverse transcription–polymerase chain reaction (RT-PCR), enzyme immunoassay, and immunofluorescence in cultured FLS, and by immunohistochemical analysis in synovial tissue. The expression and function of the potential VIP receptors in FLS were studied by RT-PCR, determination of intracellular cAMP production, cell membrane adenylate cyclase (AC) activity, and interleukin-6, CCL2, and CXCL8 production in response to VIP or specific agonists and antagonists.**

Results. **VIP expression was detected in human FLS at the messenger RNA and protein levels, and it was significantly decreased in RA FLS compared with OA FLS. VIP receptor type 1 ($VPAC_1$) was the dominant AC-coupled receptor in OA FLS, in contrast with RA FLS, in which $VPAC_2$ was dominant. Tumor necrosis factor α–treated OA FLS reproduced the VIP and VPAC receptor expression pattern of RA FLS. The antagonistic effects of VIP on FLS proinflammatory factor production were reproduced by $VPAC_1$- and $VPAC_2$-specific agonists in OA FLS and RA FLS, respectively.**

Conclusion. **VIP expression is down-regulated in RA and in tumor necrosis factor α–treated FLS, suggesting that down-regulation of this endogenous antiinflammatory factor may contribute to the pathogenesis of RA. In RA FLS, $VPAC_2$ mediates the antiinflammatory effects of VIP, suggesting that $VPAC_2$ agonists may be an alternative to VIP as antiinflammatory agents.**

The vasoactive intestinal peptide (VIP)/pituitary adenylate cyclase–activating peptide (PACAP) system consists of 2 peptides and 3 receptors: VIP receptor type 1 ($VPAC_1$), $VPAC_2$, and PACAP type 1 (PAC_1) receptor. These receptors belong to family 2 of the G protein–coupled receptors, which in recent years have shown remarkable antiinflammatory and immunomodulatory properties (1–3). $VPAC_1$ is constitutively expressed in macrophages and lymphocytes and binds VIP and PACAP with equal affinity (1,3). $VPAC_2$ has also been described in lymphocytes and macrophages as an inducible receptor after T cell receptor triggering or lipopolysaccharide (LPS) stimulation (1,3). The best-characterized effects of VIP/PACAP on immune cells are mediated by the adenylate cyclase (AC) pathway coupled to these receptors. Finally, the PAC_1 receptor is the PACAP-specific receptor, although at high concentrations VIP is a heterologous ligand (4). Endogenous VIP and PACAP can be produced by both neural and immune cells, but their regulation and participation in the pathogenesis of human inflammation are not known.

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Different studies of murine models of arthritis and other inflammatory diseases have consistently demonstrated the potential of exogenous VIP to interfere with both adaptive and innate components of autoimmune inflammatory responses (5–10). Although clinical trials of VIP or related neuropeptides in human inflammatory diseases have not been performed, our previous studies demonstrated that exogenous VIP modulates different proinflammatory pathways ex vivo in human rheumatoid arthritis (RA) synovial cells (11,12). The detection of several neuropeptides, including VIP, in human arthritic joints suggests that endogenous VIP might play a regulatory role in response to joint inflammation (13,14). Results of previous studies point to variable inflammation-regulated expression of VIP as well as the different VIP receptors in murine cells, but information regarding human inflammatory cells is scarce (15).

Fibroblast-like synoviocytes (FLS) are an abundant synovial cell population and play essential roles in RA pathogenesis by contributing to inflammation and joint destruction (16,17). These cells produce a variety of mediators such as chemokines, cytokines, and cell-surface molecules that contribute to the chronic inflammatory process and to bone and cartilage destruction. According to previous studies by our group and by other investigators, VIP antiinflammatory signaling is functional in human FLS, but the ability of these cells to synthesize VIP or to regulate VIP or VIP receptor expression under inflammatory conditions has not been investigated (11,12). Alternatively, because VIP and related neuropeptides are short-lived peptides, identification of specific antiinflammatory signaling receptors in relevant human cellular models is needed to develop antiinflammatory VIP agonists. Therefore, we studied the expression and regulation of VIP and PACAP neuropeptides and their potential receptors in human osteoarthritis (OA) FLS and RA FLS.

PATIENTS AND METHODS

Patients and FLS cultures. Synovial tissue samples were obtained from 9 patients with RA and 7 patients with OA at the time of knee prosthetic replacement surgery. All patients with RA fulfilled the American College of Rheumatology (formerly, the American Rheumatism Association) 1987 revised criteria for the diagnosis of RA (18). The study was performed according to the recommendations of the Declaration of Helsinki and was approved by the ethics committee of the Hospital 12 de Octubre. FLS cultures were established from homogenized synovium in 10% fetal calf serum–Dulbecco's modified Eagle's medium. The purity of the FLS used in the experiments was ≥95% by flow cytometric analysis. FLS were used between passages 3 and 8. In some experiments, FLS were cultured in the presence or absence of 10 n*M* tumor necrosis factor α (TNFα) (Genzyme, Cambridge, UK) or 10 n*M* VIP (Neosystems, Strasbourg, France), $VPAC_1$ selective agonist [K^{15}, R^{16}, L^{27}]VIP(1–7)/GRF(8–27) (19), $VPAC_1$ antagonist [D-Phe^2, K^{15}, R^{16}, L^{27}]VIP(1–7)/GRF(8–27) (20), $VPAC_2$ selective agonist (RO 25-1553) (21), or PAC_1 selective agonist (maxadilan) (22) for 24 hours (GL Biochem Ltd., Shanghai, China).

Culture supernatants were harvested and stored at −20°C for enzyme-linked immunosorbent assay (ELISA) or enzyme immunoassay (EIA). RNA was also obtained from FLS cultures (1×10^6 cells/ml) using the Ultraspec RNA Isolation System (Biotecx, Houston, TX) and was stored at −80°C.

For membrane preparation, FLS were harvested with a rubber policeman and pelleted by low-speed centrifugation. The supernatant was discarded and the cells lysed in 1 m*M* $NaHCO_3$ and immediately frozen in liquid nitrogen. After thawing, the lysate was first centrifuged at 800*g* for 10 minutes. The supernatant was further centrifugated at 20,000*g* for 15 minutes. The pellet was resuspended in 1 m*M* $NaHCO_3$ and used immediately as a crude membrane preparation.

Determination of VIP and VIP receptor expression in FLS. VIP, PACAP, and $VPAC_1$, $VPAC_2$, or PAC_1 receptor subtype messenger RNA (mRNA) expression in cultured FLS was analyzed by reverse transcription–polymerase chain reaction (RT-PCR). Total RNA (2 μg) was reverse transcribed using 6-μg random hexamer primers and 200 units SuperScript II reverse transcriptase in the buffer supplied, with 10 m*M* dithiothreitol and 40 units RNase OUT (all from Invitrogen, Carlsbad, CA) and 0.5 m*M* dNTP. Reverse transcriptase products (2 μl) were PCR-amplified with specific primers for β-actin, VIP, PACAP, and the $VPAC_1$, $VPAC_2$, or PAC_1 receptor subtypes (Table 1). The PCR products were analyzed in agarose gels.

Single-step relative quantitative RT-PCR analysis was also performed using SYBR Green PCR Master Mix and an RT-PCR kit (Applied Biosystems, Foster City, CA). Briefly, reactions were performed in 20 μl with 50 ng RNA, 10 μl 2× SYBR Green PCR Master Mix, 6.25 units SuperScript II reverse transcriptase, 10 units RNase OUT (Invitrogen), and 0.1 μ*M* primers. The sequences of the primers used and the accession numbers of the genes analyzed are summarized in Table 1. For relative quantification, we compared the amount of target normalized to an endogenous reference, using the formula $2^{-\Delta\Delta C_t}$, as previously described (11).

The levels of VIP in concentrated FLS culture supernatants were also measured by EIA, using a commercial kit (Phoenix Pharmaceuticals, Karlsruhe, Germany) according to the manufacturer's instructions. The minimum detectable concentration was 0.12 ng/ml of sample, with intraassay variation and interassay variation of <5% and 14%, respectively.

To confirm VIP protein expression in cultured FLS and in synovial tissue sections, we performed immunofluorescence and immunohistochemistry studies using a specific anti-VIP rabbit polyclonal antibody (Chemicon, Temecula, CA). The specificity of this antibody has been previously reported (23). OA FLS or RA FLS were cultured onto glass coverslips,

Table 1. Primer sequences for RT-PCR amplification of the indicated genes*

Gene	GenBank accession no.	Sequence position	Primers	Sequence
β-actin	E00829	1435–1535	hβ-actin	Forward: 5′-AGAAGGATTCCTATGTGGGCG-3′ Reverse: 5′-CATGTCGTCCCAGTTGGTGAC-3′
VIP	NM36634	538–638	hVIP	Forward: 5′-ACGTCACTCAGATGCAGTCTTCAC-3′ Reverse: 5′-TCGTCCTCTTTCCATTCAGAATT-3′
PACAP	NM001117	502–1078	hPACAP	Forward: 5′-AAACAAAGGACGCCGAATAG-3′ Reverse: 5′-AGACTCACTGGGAAAGAATGC-3′
$VPAC_1$	NM004624	2120–2220	$hVPAC_1$	Forward: 5′-CCCCTGGGTCAGTCTGGTG-3′ Reverse: 5′-GAGACCTAGCATTCGCTGGTG-3′
$VPAC_2$	NM003382	3672–3772	$hVPAC_2$	Forward: 5′-TGGTTGGGTGTGGGCAG-3′ Reverse: 5′-GGAGAAACCAGTCAGCTCCG-3′
PAC_1	NM001118	966–1365	$hPAC_1$	Forward: 5′-GCCTGTACCTCTTCACTCGC-3′ Reverse: 5′-CTTTCCCTTTTGCTGACATTC-3′

* RT-PCR = reverse transcription–polymerase chain reaction; VIP = vasoactive intestinal peptide; PACAP = pituitary adenylate cyclase–activating peptide; $VPAC_1$ = VIP receptor type 1; PAC_1 = PACAP type 1.

fixed with 4% paraformaldehyde, and permeabilized for 10 minutes with 0.5% Triton X-100 in phosphate buffered saline at room temperature. The coverslips were incubated with anti-VIP antibody for 30 minutes at 37°C, followed by a 1-hour incubation with an Alexa Fluor 594 goat anti-rabbit IgG antibody (Invitrogen). Coverslips were counterstained with 1 μg/ml 4′,6-diamidino-2-phenylindole to visualize nuclear bodies, mounted, and examined under a fluorescence microscope.

Tissue specimens from patients with OA and patients with RA were snap-frozen in OCT compound and stored at –80°C. Frozen sections (8 μm) were fixed with 4% paraformaldehyde. Endogenous peroxidase was quenched in 3% H_2O_2 in methanol for 20 minutes. Cryosections were incubated with anti-VIP rabbit polyclonal antibody, and immunostaining was performed following a standard indirect avidin–biotin–horseradish peroxidase method (Vector, Burlingame, CA). Color was developed with diaminobenzidine (Vector), and slides were counterstained in Gill's hematoxylin.

Determination of cAMP and AC activity in FLS cultures. Levels of cAMP were determined by means of an EIA kit (Cayman Chemical, Ann Arbor, MI). OA FLS and RA FLS were cultured in the presence of 100 n*M* VIP, $VPAC_1$ agonist, or $VPAC_2$ agonist for 24 hours. The cells were then homogenized in 5% trichloroacetic acid. After centrifugation, cAMP levels in the supernatants were measured according to the manufacturer's instructions. The protein concentration was determined by the Bradford method, using bovine serum albumin as a standard. Results are expressed in picomoles of cAMP per microgram of protein.

AC activity was measured as described elsewhere (24), with minor modifications. Briefly, membranes (4.5 μg) were incubated with 1.5 m*M* ATP, 10*M* $MgSO_4$, an ATP-regenerating system (7.5 mg/ml phosphocreatine and 1 mg/ml creatine kinase), 2 m*M* 3-isobutyl-1-methyl xanthine, 2 m*M* EDTA, and 2 mg/ml bacitracin, and the substances were tested in 0.1 ml of 25 m*M* triethanolamine HCl buffer (pH 7.4). After 15 minutes at 30°C, the reaction was stopped by heating the mixture for 3 minutes. After refrigeration, 0.2 ml of alumina slurry was added, and the suspension was centrifuged. The supernatant was obtained for assay of cAMP (25).

Cytokine and chemokine determinations. The amounts of interleukin-6 (IL-6), chemokine (CC motif) ligand 2 (CCL2; monocyte chemoattractant protein 1 [MCP-1]), and chemokine (CXC motif) ligand 8 (CXCL8; IL-8) in the supernatants of FLS cultures treated with 100 n*M* VIP, $VPAC_1$ agonist, or $VPAC_2$ agonist for 24 hours were determined with a human capture ELISA, as previously described (11). Both the intraassay and interassay variability for cytokine and chemokines determination were <5%.

Statistical analysis. Results are expressed as the mean ± SEM. The significance of the results was analyzed using Student's 2-tailed *t*-test. *P* values less than 0.05 were considered significant.

RESULTS

VIP expression by OA FLS and RA FLS. Constitutive VIP mRNA expression was uniformly detected by electrophoretic analysis of RT-PCR products in all 6 OA FLS lines, whereas it was detected in only 3 of 9 RA FLS lines (Figure 1A, top, and results not shown). The identity of the PCR products was verified by sequencing them, and the integrity of cDNA templates was verified by β-actin RT-PCR. PACAP mRNA expression was detected in neither OA FLS nor RA FLS, whereas it was detected in a human tonsil specimen used as positive control (data not shown). To confirm lower expression of VIP mRNA by RA FLS, we performed a relative quantitative real-time RT-PCR analysis of VIP mRNA expression in all FLS cell lines. Ten-fold lower VIP mRNA expression was observed in RA FLS compared with OA FLS (Figure 1A, bottom).

The expression of VIP protein by FLS was confirmed by immunofluorescence labeling of cultured FLS,

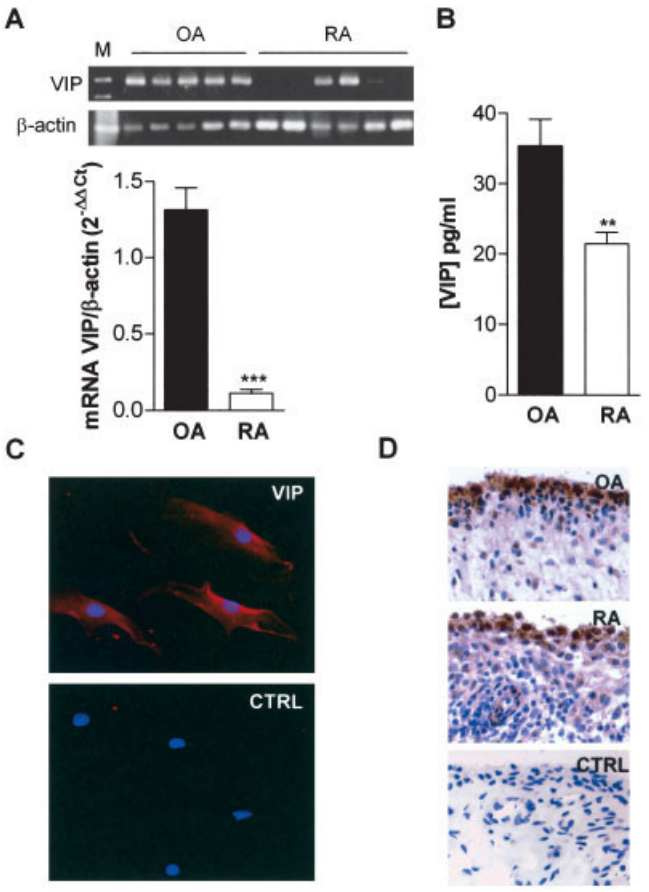

Figure 1. Differential expression of vasoactive intestinal peptide (VIP) by osteoarthritis (OA) and rheumatoid arthritis (RA) fibroblast-like synoviocytes (FLS). **A,** VIP mRNA expression in OA FLS and RA FLS. **Top,** Electrophoretic analysis of reverse transcription–polymerase chain reaction (RT-PCR) products. M = 10-bp ladder. **Bottom,** VIP mRNA expression in OA FLS and RA FLS as measured by relative quantitative real-time RT-PCR and corrected for mRNA expression of β-actin in each sample (see Patients and Methods). Values are the mean and SEM of triplicate determinations (n = 7 OA FLS and 9 RA FLS lines). *** = $P < 0.001$ versus OA FLS. **B,** Presence of VIP in the supernatants of OA and RA FLS cultures under basal conditions, determined by enzyme immunoassay after 24 hours of culture. Values are the mean and SEM results from 2 independent experiments performed in triplicate (n = 7 OA FLS and 9 RA FLS lines). ** = $P < 0.01$ versus OA FLS. **C,** Immunofluorescence labeling of VIP in cultured OA FLS (red). Cells were counterstained with 4′,6-diamidino-2-phenylindole (blue nuclei). **D,** Immunoperoxidase staining of VIP in OA and RA synovial sections (hematoxylin counterstained). CTRL = control. (Original magnification × 450 in **C**; × 300 in **D**.)

which displayed clear VIP cytoplasmic immunolabeling (Figure 1C). Immunohistochemical analysis of OA and RA synovial membrane sections also showed VIP immunoperoxidase labeling of lining synoviocytes (Figure 1D). Because these techniques do not allow for quantitative analyses, VIP protein expression was determined in the supernatants of OA FLS and RA FLS cultures by EIA (Figure 1B). This analysis confirmed VIP protein expression and its secretion by FLS cultures, with significantly lower levels in RA FLS compared with OA FLS. Therefore, these data pointed to a deficit of VIP mRNA and protein expression in RA FLS compared with OA FLS.

VIP/PACAP receptor expression and function in OA FLS and RA FLS. To investigate the potentially functional receptors for VIP in these cells, we first performed an RT-PCR analysis of the expression of PAC_1, $VPAC_1$, and $VPAC_2$ mRNA in OA FLS and RA FLS. PAC_1 receptor mRNA was detected in human tonsil–positive control but not in OA FLS and RA FLS lines (results not shown). In contrast, $VPAC_1$ and $VPAC_2$ mRNA expression was variably detected by RT-PCR in OA FLS and RA FLS (Figures 2A and B, top). Relative quantitative real-time RT-PCR analysis showed significantly decreased $VPAC_1$ mRNA expression in RA FLS compared with that in OA FLS (Figure 2A, bottom) and, in contrast, significantly increased $VPAC_2$ mRNA expression in RA FLS compared with that in OA FLS (Figure 2B, bottom).

Although cAMP-independent signaling (phospholipase C, phospholipase D, $[Ca^{2+}_i]$, etc.) is associated with $VPAC_1$ and $VPAC_2$, cAMP production represents the most prominent signaling pathway of VIP in VPAC receptor–mediated biologic responses (26). Thus, the functional activity of VIP/PACAP receptors was studied by determining AC activity in OA FLS membranes and RA FLS membranes (Figure 2C) and the intracellular levels of cAMP in intact cells after treatment with VIP or $VPAC_1$ or $VPAC_2$ agonists (Table 2). The mean ± SEM basal values for AC activity in OA FLS and RA FLS were 4.2 ± 0.4 and 7.4 ± 4.0 pmoles cAMP/minute/mg protein, respectively. VIP stimulated AC activity in a dose-dependent manner in OA FLS and RA FLS. The order of potency in each membrane type was 4.2 n*M* and 3 n*M*, respectively (Table 3). In OA FLS membranes, the potency for each VIP/PACAP receptor agonist was $VPAC_1$ agonist > $VPAC_2$ agonist > PAC_1 agonist; in RA FLS membranes, the potency was $VPAC_2$ agonist > $VPAC_1$ agonist > PAC_1 agonist (Figure 2C, top, and Table 3). In intact cells, stimulation of FLS with 100 n*M* $VPAC_1$ agonist or $VPAC_2$ agonist significantly increased intracellular levels of cAMP in OA FLS and RA FLS, respectively (Table 2). Stimulation with maxadilan, the specific agonist for PAC_1 receptor, was much

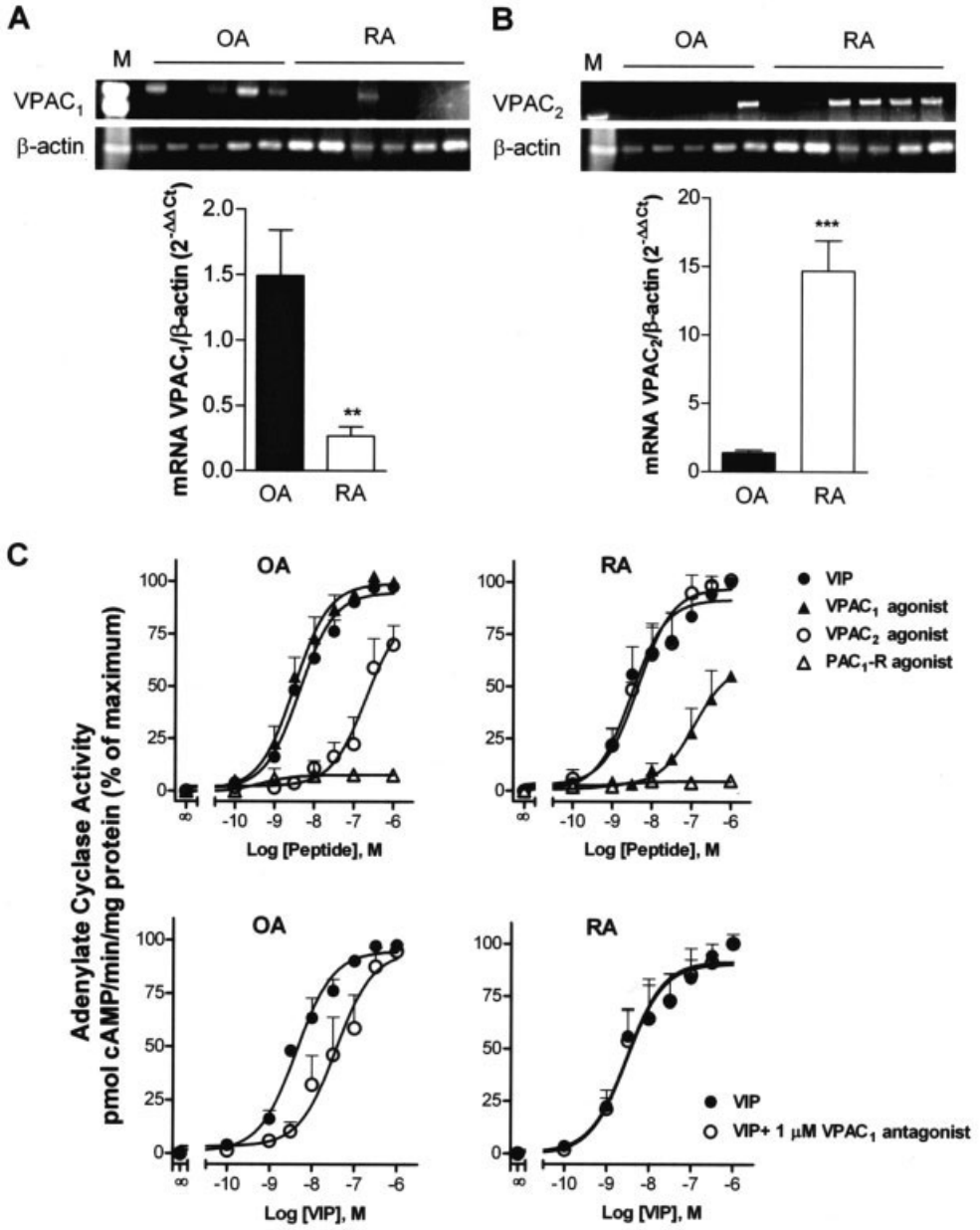

Figure 2. Differential VIP receptor expression and function in OA and RA FLS. **A** and **B,** VIP receptor type 1 ($VPAC_1$) and $VPAC_2$ mRNA expression in OA FLS and RA FLS. **Top,** Electrophoretic analysis of RT-PCR products. **Bottom,** $VPAC_1$ **(A)** and $VPAC_2$ **(B)** mRNA expression in OA FLS and RA FLS as measured by relative quantitative real-time RT-PCR and corrected for mRNA expression of β-actin in each sample. Values are the mean and SEM of triplicate determinations (n = 7 OA FLS and 9 RA FLS). ** = $P < 0.01$; *** = $P <$ 0.001, versus OA FLS. **C,** Effect of VIP and specific agonists on adenylate cyclase activity in OA FLS and RA FLS membranes. **Top,** Dose-effect curves of VIP, $VPAC_1$ agonist, $VPAC_2$ agonist, and pituitary adenylate cyclase–activating peptide type 1 receptor (PAC_1-R) agonist. **Bottom,** VIP dose-effect curve in the absence or presence of 1 μM $VPAC_1$ antagonist. Values are the mean and SEM results from duplicate determinations (n = 3–4 different OA FLS and RA FLS cell lines). See Figure 1 for other definitions.

Table 2. Effect of VIP, $VPAC_1$ agonist, and $VPAC_2$ agonist on intracellular cAMP levels in OA FLS and RA FLS*

	OA	RA
Basal	0.59 ± 0.24	3.95 ± 0.87
VIP, 100 n*M*	13.8 ± 5.20†	10.5 ± 2.31†
$VPAC_1$ agonist, 100 n*M*	14.0 ± 8.28†	5.93 ± 1.54
$VPAC_2$ agonist, 100 n*M*	5.75 ± 3.48	10.2 ± 3.04†

* Values are pmoles cAMP/μg of protein. Results are the mean ± SEM of duplicate determinations in 3 different fibroblast-like synoviocyte (FLS) lines. VIP = vasoactive intestinal peptide; $VPAC_1$ = VIP receptor type 1; OA = osteoarthritis; RA = rheumatoid arthritis.
† $P < 0.05$ versus basal.

Table 3. Potency of VIP, $VPAC_1$ agonist, $VPAC_2$ agonist, and PAC_1 agonist in adenylate cyclase stimulation in OA FLS and RA FLS membranes*

	OA	RA
VIP	4.2 ± 0.78	3 ± 1.2
$VPAC_1$ agonist	3.1 ± 0.36	120 ± 13.5†
$VPAC_2$ agonist	200 ± 7†	4.4 ± 1.45
PAC_1 agonist	>1,000†	>1,000†

* Values (50% maximum response concentration) are expressed in n*M* and correspond to the agonist concentration that induced a half-maximal response. Results are the mean ± SEM of at least 3 determinations in 3 different FLS lines. PAC_1 = pituitary adenylate cyclase–activating peptide type 1 receptor (see Table 2 for other definitions).
† The maximal response was lower than that of VIP (partial agonist).

less efficient than was stimulation with VIP, corroborating our negative data on PAC_1 receptor expression in FLS.

In order to confirm the results obtained with receptor agonists, FLS were treated with VIP in the presence of a specific $VPAC_1$ antagonist. The $VPAC_1$ antagonist was able to inhibit the effect of VIP on AC activity in OA FLS (Ki = 128 n*M*) but was incapable of inhibiting it in RA FLS (Figure 2C, bottom).

To determine whether $VPAC_1$ or $VPAC_2$ was also dominant, based on our previous observation of VIP inhibition of proinflammatory factor synthesis in FLS (11), we analyzed this effect in OA FLS and RA FLS treated with $VPAC_1$ or $VPAC_2$ agonist. VIP and $VPAC_1$ agonist significantly inhibited IL-6 release by

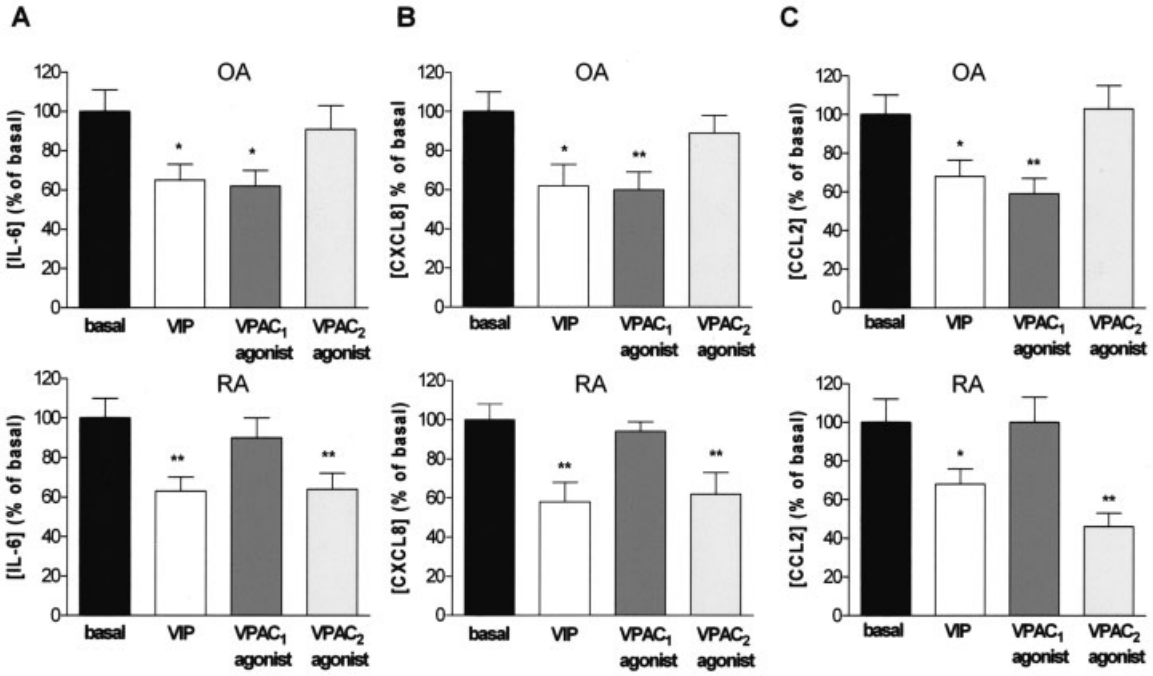

Figure 3. Effect of VIP, VIP receptor type 1 ($VPAC_1$) agonist, and $VPAC_2$ agonist on interleukin-6 (IL-6) and chemokine production in FLS. The supernatants of OA FLS and RA FLS cultures under basal unstimulated conditions and after treatment with 10 n*M* VIP, $VPAC_1$ agonist, or $VPAC_2$ agonist for 24 hours were collected, and levels of IL-6 **(A)**, CXCL8 **(B),** and CCL2 **(C)** were determined by enzyme-linked immunosorbent assay. Values are the mean and SEM results from 3 independent experiments performed in triplicate, which included FLS lines from 3 patients with OA and 4 patients with RA. * = $P < 0.05$; ** = $P < 0.01$, versus basal. See Figure 1 for other definitions.

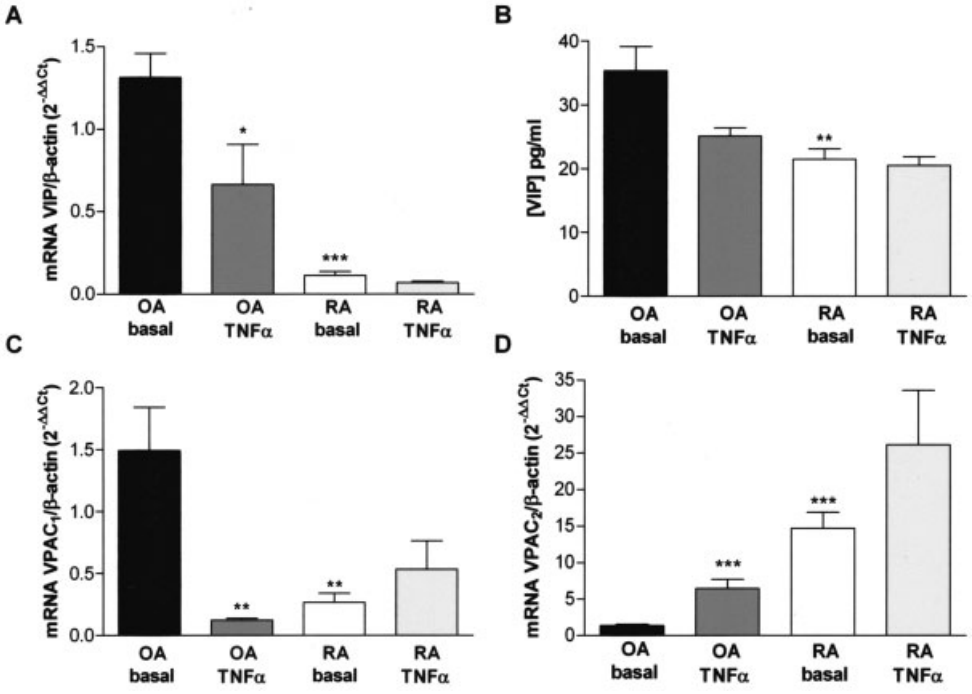

Figure 4. VIP and VIP receptor expression in FLS treated with 10 n*M* tumor necrosis factor α (TNFα) for 24 hours. **A,** Expression of VIP mRNA in OA and RA FLS was measured by relative quantitative real-time RT-PCR and corrected for mRNA expression of β-actin in each sample. **B,** The presence of VIP in supernatants from OA FLS and RA FLS cultures in basal and TNFα-stimulated conditions was determined by enzyme immunoassay. **C** and **D,** Expression of mRNA for VIP receptor type 1 ($VPAC_1$) (**C**) and $VPAC_2$ (**D**) in OA FLS and RA FLS under basal and TNFα-stimulated conditions was measured by relative quantitative real-time RT-PCR and corrected for mRNA expression of β-actin in each sample. Values are the mean and SEM results from triplicate determinations (n = 7 OA FLS lines and 8 RA FLS lines). $* = P < 0.05$; $** = P < 0.01$; $*** = P < 0.001$, versus OA basal. See Figure 1 for other definitions.

OA FLS cultures, whereas VIP and $VPAC_2$-specific agonist had the same effect in RA FLS (Figure 3A). VIP and $VPAC_1$ agonist also significantly decreased CXCL8 (IL-8) and CCL2 (MCP-1) chemokine synthesis in OA FLS, whereas VIP and $VPAC_2$ agonist significantly decreased it in RA FLS (Figures 3B and C).

Taken together, these results are consistent with $VPAC_1$ receptor expression and functional coupling to AC and antiinflammatory effects in OA FLS, whereas $VPAC_2$ is the receptor expressed and coupled to AC and antiinflammatory effects in RA FLS.

Expression of VIP and VIP receptors in TNFα-treated FLS. To obtain further evidence of the regulation of VIP and VIP receptor expression in FLS in the context of inflammation, we studied VIP mRNA and protein expression in OA FLS and RA FLS after treatment with the proinflammatory cytokine TNFα. TNFα induced a significant reduction of VIP mRNA expression compared with basal levels in OA FLS (Figure 4A). At the protein level, a similar trend was observed, although the response was poorer compared with the observed mRNA changes (Figure 4B). The inhibitory effect of TNFα was specific, because treatment with LPS had the opposite effect, strongly inducing VIP expression in both OA FLS and RA FLS (results not shown).

Treatment of OA FLS with TNFα significantly reduced mRNA expression of $VPAC_1$ receptor, to a level similar to that detected constitutively in RA FLS. In contrast, treatment with TNFα did not significantly modify $VPAC_1$ mRNA expression compared with its basal levels in RA FLS (Figure 4C). Furthermore, relative quantitative real-time RT-PCR analysis showed that TNFα induced $VPAC_2$ receptor mRNA expression in OA FLS compared with basal levels (Figure 4D). Taken together, these results show that TNFα treatment

of OA FLS induced a pattern of VIP and VIP receptor expression similar to the constitutive expression observed in RA FLS.

DISCUSSION

An abundant number of studies have consistently demonstrated potent immunomodulatory and antiinflammatory effects of exogenous administration of VIP (and related neuropeptides) in a variety of animal models of inflammatory disease, providing a solid basis for its potential use as a therapeutic agent (1–3,6–9,27). Several studies in the murine model of collagen-induced arthritis have shown that VIP or PACAP potently inhibits inflammatory and autoimmune components, protecting the joints from structural damage (5,6,28). Our previous findings in human synovial cells confirm some antiinflammatory effects of VIP under both constitutive and TNFα- or Toll-like receptor 4–stimulated conditions (12,29). A less developed concept is the potential endogenous participation of these neuropeptides in the pathogenesis of inflammation, either as endogenous mediators counterbalancing proinflammatory signaling or as permissive factors, if they are down-regulated during inflammatory responses.

Our present data using human FLS as a cellular model are the first to demonstrate that VIP can be produced by human fibroblasts, and that VIP expression is down-regulated in a chronic inflammatory condition or after short-term exposure to the proinflammatory cytokine TNFα. In this model, down-regulation of VIP under proinflammatory conditions would facilitate proinflammatory signaling in RA FLS. Because human synovial tissue is innervated by both sensory and sympathetic nerves, potential changes in the neural expression of neuropeptides under conditions of painful inflammation could also contribute to local VIP synthesis. Previous data suggest that neural VIP expression is also down-regulated in RA synovium, resulting in a global deficit of VIP production (30,31).

Our present study contributes to whole mapping of the VIP/PACAP peptides and the receptor family in RA FLS and OA FLS. A striking observation is the switch from the dominance of constitutive $VPAC_1$ expression and function in control OA FLS to dominance of inducible $VPAC_2$ in either RA FLS or ex vivo TNFα-treated FLS. According to our findings, these seem to be the only expressed and signaling receptors in FLS; furthermore, the antiinflammatory effects of VIP on IL-6 or chemokine synthesis in this cellular model were fully reproduced by specific $VPAC_1$ or $VPAC_2$ agonists, in a disease-specific manner. Previous data reported by Takeba et al (32) had already suggested that $VPAC_2$ was the only receptor expressed and functional in FLS, based on data limited to RA FLS. Previous data in rodent and human myeloid and lymphoid cells have also shown a different distribution and regulation of $VPAC_1$ and $VPAC_2$ (33,34). Monocytes and T cells also constitutively express $VPAC_1$, whereas $VPAC_2$ is inducible by specific cell activation (35–37). $VPAC_1$ receptor has also been preferentially detected in CD34+,CD38− primitive hematopoietic stem cells (38). All of these data indicated that in several systems, $VPAC_2$ could represent a marker of cell activation or differentiation, explaining its predominant presence in RA FLS or TNFα-treated OA FLS.

The genomic organization of human $VPAC_1$ and $VPAC_2$ is very similar, but little is known about the transcriptional factors that control specific VPAC receptor expression in the different tissues studied (26). Some transcriptional repressors have been described in the case of $VPAC_1$, but little is known about $VPAC_2$ transcriptional regulation (39,40). Therefore, the inflammation-related mechanisms involved in the switch of VPAC receptor expression observed in FLS as well as in other inflammatory cell types remain to be elucidated.

VPAC receptor–associated signal transduction involves the stimulation of AC, which triggers a protein kinase A pathway. This represents the major signaling pathway in different biologic responses. Several studies have highlighted the therapeutic potential of cAMP agonists for the treatment of arthritis (41,42). Our data confirm the functional coupling of VIP receptors to AC in OA FLS and RA FLS. Specific agonists for each receptor subtype are equivalent to VIP in terms of activating the dominant receptor ($VPAC_1$ or $VPAC_2$) in OA FLS or RA FLS. This fact was confirmed by studies of AC activity, and, more importantly, by cytokine and chemokine synthesis. Because RA appears to be a more rational target disease than OA for the development of antiinflammatory neuropeptides, the use of stable $VPAC_2$ receptor agonists is an attractive alternative to the use of parenteral short-lived neuropeptides (43). In this regard, the $VPAC_2$ agonist used in this study (RO25-1553) was recently tested in patients with asthma, without significant toxicity (44).

In summary, our study shows inflammation-associated down-regulation of the antiinflammatory neuropeptide VIP in resident RA fibroblasts and dissects the regulation of VIP functional receptors in this

context, underscoring $VPAC_2$ receptor as a potential target of therapy in RA.

AUTHOR CONTRIBUTIONS

Dr. Juarranz had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Juarranz, Gomariz.
Acquisition of data. Juarranz, Gutiérrez-Cañas, Santiago, Carrión.
Analysis and interpretation of data. Juarranz, Pablos, Gomariz.
Manuscript preparation. Juarranz, Pablos, Gomariz.
Statistical analysis. Juarranz, Gomariz.

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