Biochimica et Biophysica Acta 1823 (2012) 1676–1685

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Vasoactive intestinal peptide (VIP) inhibits human renal cell carcinoma proliferation

Eva Vacas [a], Ana B. Fernández-Martínez [b], Ana M. Bajo [a], Manuel Sánchez-Chapado [c,d], Andrew V. Schally [e,f,g,h], Juan C. Prieto [a,*], María J. Carmena [a]

[a] Department of Biochemistry and Molecular Biology, University of Alcalá, 28871 Alcalá de Henares, Spain
[b] Department of Physiology, University of Alcalá, 28871 Alcalá de Henares, Spain
[c] Department of Surgery, University of Alcalá, 28871 Alcalá de Henares, Spain
[d] Department of Urology, Príncipe de Asturias Hospital, 28871 Alcalá de Henares, Spain
[e] Veterans Administration Medical Center, Miami, FL 33125, USA
[f] Department of Pathology, University of Miami Miller School of Medicine, Miami, FL 33125, USA
[g] Department of Medicine, Division of Endocrinology, University of Miami Miller School of Medicine, Miami, FL 33125, USA
[h] Department of Medicine, Division of Hematology-Oncology, University of Miami Miller School of Medicine, Miami, FL 33125, USA

ARTICLE INFO

Article history:
Received 13 February 2012
Received in revised form 29 May 2012
Accepted 14 June 2012
Available online 21 June 2012

Keywords:
VIP
FPRL-1
cAMP
PI3-K
Proliferation
RCC

ABSTRACT

Clear renal cell carcinoma (cRCC) is an aggressive and fatal neoplasm. The present work was undertaken to investigate the antiproliferative potential of vasoactive intestinal peptide (VIP) exposure on non-tumoral (HK2) and tumoral (A498, cRCC) human proximal tubular epithelial cell lines. Reverse transcription and semiquantitative PCR was used at the VIP mRNA level whereas enzyme immunoanalysis was performed at the protein level. Both renal cell lines expressed VIP as well as VIP/pituitary adenylate cyclase-activating peptide (VPAC) receptors whereas only HK2 cells expressed formyl peptide receptor-like 1 (FPRL-1). Receptors were functional, as shown by VIP stimulation of adenylyl cyclase activity. Treatment with 0.1 μM VIP (24 h) inhibited proliferation of A498 but not HK2 cells as based on a reduction in the incorporation of [^{3}H]-thymidine and BrdU (5′-Br-2′-deoxyuridine), PCNA (proliferating-cell nuclear antigen) expression and STAT3 (signal transducer and activator of transcription 3) expression and activation. $VPAC_1$-receptor participation was established using JV-1-53 antagonist and siRNA transfection. Growth-inhibitory response to VIP was related to the cyclic adenosine monophosphate (cAMP)/exchange protein directly activated by cAMP (EPAC)/phosphoinositide 3-kinase (PI3-K) signaling systems as shown by studies on adenylate cyclase stimulation, and using the EPAC-specific compound 8CPT-2Me-cAMP and specific kinase inhibitors such as H89, wortmannin and PD98059. The efficacy of VIP on the prevention of tumor progression was confirmed in vivo using xenografted athymic mouse. These actions support a potential role of this peptide and its agonists in new therapies for cRCC.

1. Introduction

Renal cell carcinoma (RCC) is a tumor that mainly arises from renal tubular epithelium [1] and represents about 90% of all renal malignancies [2]. RCC is the most frequent and lethal malignant tumor of the kidney and the third most common malignancy in the field of urological oncology [3]. Surgical resection of the tumor is considered the only curative treatment [4]. In fact, metastatic RCC is refractory to chemotherapy due to the expression of multidrug resistance transporters in proximal tubules [1]. The response to radiotherapy is also negative, whereas cytokine-based immunotherapy elicits some benefits in about 20% of patients [5]. Molecular markers with potential diagnostic and prognostic value are not clearly defined in RCC as tumor targets at which a new therapy can be aimed have not been defined [6]. One approach to control RCC is the inhibition of cell growth, but the results available are limited. Thus, effective therapies for advanced RCC are needed to improve patient prognosis.

Signal transducer and activator of transcription (STAT) factor is an important point of convergence for various signaling pathways that are activated in the malignant transformation of many human tissues [7]. STATs are a family of transcription factors that are often stimulated in response to the activation of receptors for cytokines, chemokines and growth factors through Janus kinases (JAKs) as well as an increasing number of G protein-coupled receptors (GPCRs) [8,9]. STAT3 is the most studied STAT protein. Constitutive activation of STAT3 has been observed in many types of cancer [10]. STAT3 is a positive regulator of tumor cell proliferation, cell motility and anti-apoptotic genes [11,12]. The activation of STAT3 has been associated with progression of RCC and poor survival [13], but its role as a prognostic marker for this disease remains elusive as studied by an assessment of its mRNA expression [14].

Vasoactive intestinal peptide (VIP), a neuropeptide widely expressed throughout the body, is a member of the pituitary adenylate cyclase-activating polypeptide (PACAP)/secretin/glucagon family of peptides. VIP exerts a wide range of biological effects acting through PAC_1, $VPAC_1$

* Corresponding author at: Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Alcalá, Campus Universitario, 28871 Alcalá de Henares, Spain. Tel.: +34 918854527; fax: +34 918854585.
E-mail address: juancarlos.prieto@uah.es (J.C. Prieto).

0167-4889/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamcr.2012.06.018

and $VPAC_2$ receptors [15,16]. $VPAC_1$ and $VPAC_2$ receptors are G-protein coupled heptahelical receptors that lead mainly to the activation of the cAMP signal-transduction system but are also involved in other intracellular pathways [12,16]. Recently, it has been reported that VIP is a functional ligand for formyl peptide receptor-like 1 (FPRL-1) in human monocytes [17]. While cAMP signaling has classically been associated with the activation of protein kinase A (PKA), many effects of VIP do not rely solely upon PKA signaling. In this context, cAMP also directly regulates the exchange protein directly activated by cAMP (EPAC), a guanine nucleotide exchange factor (GEF) that is a crucial mediator of PKA-independent cAMP signaling [17,18].

Previous observations on PACAP effects in renal cells have provided some evidence that this VIP-related peptide may induce renoprotective effects. Thus, PACAP protects against oxidative stress in primary rat kidney culture [19]. Moreover, PACAP behaves as a cytoprotective agent in multiple myeloma and other kidney diseases [20]. These features together with observations on the inhibitory effects of VIP on cell proliferation in tumors such as neuroblastoma [21] or hepatocellular carcinoma [22] prompted us to study the action of VIP in renal cell carcinoma. An inhibitory effect of VIP on the growth and progression of this carcinoma could be considered to develop new therapies. Here, we used human renal cell cultures (non-neoplastic HK2 and neoplastic A498 cells (clear cell RCC) in order to characterize the VIP-receptor system and its possible relation with cellular processes typically associated with cell proliferation and cancer progression.

2. Materials and methods

2.1. Cell culture

The human renal proximal tubular epithelial cell line HK2 and the human renal carcinoma cell line A498 were purchased from the American Type Culture Collection ATCC (Rockwell, MD, USA). HK2 cells were cultured in complete DMEM/F12 medium (Invitrogen, Barcelona, Spain), ITS and L-glutamine. A498 cells were cultured in Eagle's Modified Medium. Both media were supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin/amphoterycin B. Cell culture was performed in a humidified 5% CO_2 environment, at 37 °C. After cells reached 70–80% confluence, they were washed with PBS, detached with 0.25% trypsin/0.2% EDTA, and plated at 30,000–40,000 cells/cm^2. The culture medium was changed every 2 days.

2.2. RNA isolation and semiquantitative reverse transcription-PCR (RT-PCR)

HK2 and A498 cells were placed in 6-well plates ($15 \cdot 10^4$ cells per well) and maintained in their medium for 24 h. Cultured cells were stimulated with 0.1 μM VIP for different times. Then, total RNA was isolated with Tri Reagent (Sigma-Aldrich, Alcobendas, Spain) according to the instructions of the manufacturer. Two micrograms of total RNA were reverse-transcribed using 6 μg of hexamer random primer and 200 U M-MLV RT (Life Technologies, Barcelona, Spain) in the buffer supplied with the enzyme supplemented with 1.6 μg/ml oligo dT, 10 nM dithiothreitol (DTT), 40 U RNasin (Promega Madison, WI, USA), and 0.5 mM of deoxyribonucleotides (dNTPs). Two microliters of the RT reaction were used for each PCR amplification with a primer set which amplifies cDNAs for human cysteine rich protein with STAT3, VIP or β-actin. The corresponding sequences of oligonucleotide primers were: STAT3 (sense 5′-AGT GAG TAA GGC TGG GCA GA-3′ and antisense 5′-AAG GCA CCC ACA GAA ACA AC-3′), VIP (sense 5′-ACG TCA CTC AGA TGC AGT CTT CAC-3′ and antisense 5′-TGC TCC TCT TTC CAT TCA GAA TT-3′), and β-actin (sense 5′-AGA AGG ATT CCT ATG TGG GCG-3′). PCR conditions were: denaturation at 94 °C for 5 min, followed by 26–40 cycles of 95 °C for 1 min, 57 °C for 1 min, 72 °C for 1 min, and then a final cycle of 10 min at 72 °C. The signals were normalized to the β-actin gene expression level. PCR products were separated by electrophoresis and visualized in 2% agarose gels. The bands were cut from the gel, eluted, and automatically sequenced with an ABI 377 sequencer (Applied Biosystems, Alcobendas, Spain).

2.3. VIP enzyme immunoanalysis (EIA)

HK2 and A498 cells were placed in either 24-well plates (6×10^4 cells per well) to obtain extracellular secretion or 6-well plates (15×10^4 cells per well) to obtain cytosolic extracts and maintained in their medium for 24 h. Cultured cells were stimulated with 0.1 μM VIP at different times. Thereafter, extracellular secretion was collected and cells placed in 6-well-plates were lysed with 10 mM HEPES (pH 7.9), 10 mM KCL, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM phenyl methylsulfonylfluoride (PMSF), 10 μg/ml protease inhibitors, 5 mM NaF, 1 mM Na_3VO_4, and 1 mM NaN_3. Cells were kept on ice for 15 min, then 0.5% Nonidet P-40 was added and the cytosolic extract was pelleted by centrifugation at 25,000 × *g* for 40 s at 4 °C. VIP levels were quantified by EIA with a commercially available kit according to the manufacturer's instructions (Phoenix Pharmaceutical, Karlsruhe, Germany). The antiserum had 100% cross-reactivity with human VIP and VIP.

2.4. Confocal immunofluorescence microscopy

HK2 and A498 cells were placed in 24-well plates ($40–60 \times 10^3$ cells per well) on glass coverslips. The cells were fixed for 25 min at room temperature with 4% paraformaldehyde in PBS. Then, they were permeabilized with 0.1% Triton X-100 for 10 min at room temperature and blocked with 3% bovine serum albumin (BSA) for 1 h. Afterwards, cells were incubated for 1 h at room temperature with rabbit antisera against $VPAC_1$, $VPAC_2$, and PAC_1 receptors (Affinity Bioreagents, Golden, CO, USA), and antibodies to FPRL-1 receptors (Abcam, Cambridge, UK). After washing with PBS, cells were incubated with goat anti-rabbit-FITC secondary antibody (1:2000) (Alexa, Invitrogen, Barcelona, Spain) for 1 h in darkness. Cells were washed again in PBS and the coverslips were mounted with FluorSave™ Reagent (Calbiochem, San Diego, CA). Color detection was performed with a LEICA TCS-SL confocal laser scan microscope.

2.5. Protein isolation

HK2 and A498 cells ($1–2 \times 10^6$ cells in 100 mm cell culture dishes) were incubated with 0.1 μM VIP (NeoMPS, Strasbourg, France) for different time periods. The cells were washed twice with ice-cold PBS and then harvested, scraped into ice-cold PBS, and pelleted by centrifugation at 500 × *g* for 5 min at 4 °C. The cells were kept on ice for 30 min in a solution containing 20 mM Tris–HCl (pH 7.5), 1 mM EDTA, 0.5 M NaCl, 0.5% Nonidet NP-40, 2 mM PMSF, and 10 μg/ml protease inhibitors. Thereafter, the cells were pelleted by centrifugation at 4000 × *g* for 5 min at 4 °C.

2.6. Western blotting

Protein content was measured by the Bradford assay using BSA as a standard. Protein (10–50 μg) from cell lysates was solubilized in 50 mM Tris–HCl buffer (pH 6.8) containing 10% (v/v) glycerol, 3% (w/v) SDS, 0.01% bromophenol blue, and 0.7 M β-mercaptoethanol, and then heated at 95 °C for 5 min. Proteins were resolved by 10% SDS-PAGE, and transferred to nitrocellulose sheets (BioTrace/NT, Pall Corporation, East Hills, NY, USA). Rabbit monoclonal antibodies to $VPAC_1$ receptor (1:10,000) (Affinity Bioreagents), FPRL-1 receptor (1:5000) (Abcam), proliferating-cell nuclear antigen (PCNA) (Dako, Glostrup, Denmark), pSTAT3 and STAT3 (1:500) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were added followed by incubation for 1 h or overnight. After treatment for 1 h at room temperature with the corresponding HRP-labeled secondary antiserum (1:4000) (BD Biosciences, Bedford, MA, USA), the signals were detected with enhanced chemiluminescence

reagent (Amersham, Uppsala Sweden) using β-actin antibody (1:10,000) (Merck, Darmstadt, Germany) as a loading control.

2.7. Adenylyl cyclase activity

The ability of VIP and forskolin to cause elevation of cAMP levels was determined in HK2 and A498 cells. Human renal cells were placed in 6-well plates (3×10^5 cells per well) and treated with 750 μl of medium supplemented with 1% BSA, 1 mg/ml bacitracin, 0.05 mM PMSF and 0.4 mM 3-isobutyl-1-methylxanthine (IBMX). After 15 min of treatment with increasing concentrations of VIP and forskolin, the medium was removed and then 70% ethanol was added. The mixture was transferred to fresh tubes and evaporated at 60 °C. Dried samples were dissolved in 0.2 M phosphate buffer and stored at −20 °C until further assays. Afterwards, cAMP levels were measured using a competitive binding assay [23].

2.8. Cell proliferation assay with [^{3}H]-thymidine

HK2 and A498 cells were placed in 24-well plates (10^4 cells per well) and maintained in medium for 24 h. Then, they were stimulated with 0.1 μM VIP for 24 h in the presence of [^{3}H]-thymidine (0.4 μCi/ml). Afterwards, the cells were washed twice with PBS and cells were incubated with trichloroacetic acid 5%, for 15 min, at 4 °C, in the darkness. The cells were washed again with methanol and solubilized by adding 0.1 M NaOH and 0.1% SDS. Then, 0.4 ml of the cell solution was collected, placed into 4-ml scintillation vials, and counted for radioactivity.

2.9. Cell proliferation assay with 5′-Br-2′-deoxyuridine (BrdU)

HK2 and A498 cells were placed in 6-well plates (15×10^4 cells per well) and maintained in medium for 24 h. Cells were treated with VIP, PACAP38, 8-pCPT-2′-O-Me-cAMP or forskolin for 24 h. Moreover, they were cultured for 24 h with JV-1-53, a GHRH analog which behaves as a VIP antagonist [24,25] or preincubated with one of three different protein kinase inhibitors: PKA inhibitor H89 (15 min), MEK1/2 inhibitor PD98059 (1 h) or PI3-K inhibitor wortmannin (30 min), and VIP 0.1 μM, for 24 h. The cells were pulsed with 10 μM BrdU, (BD Bioscience) during the last 30 min of incubation. Afterwards, the cells were washed with PBS and fixed with ice-cold absolute ethanol, at −20 °C, for 30 min. Fixative was removed by centrifugation and the cell pellets were washed with PBS. DNA was partially denatured by incubation with 2 M HCl for 30 min at room temperature, after which the cells were washed three times with PBS containing 0.05% Tween-20 (pH 7,4) and 0.1% BSA. Cells were incubated for 30 min with 20 μl of anti-BrdU monoclonal antibody with FITC (BD Bioscience) in the dark. The cells were stained with PI staining solution (50 μg/ml PI, 10 μg/ml RNase, 1× PBS), before flow cytometric analysis. DNA distribution in the different phases of the cell cycle was analyzed with the use of the Cyflogic v. 1.2.1 program.

2.10. Transfection of $VPAC_1$-receptor siRNA

HK2 and A498 cells were seeded in medium without antibiotics in 100 mm cell culture dishes (6×10^5 cells per well) and maintained for 24 h. The next day, cells were transfected with $VPAC_1$-receptor siRNA (150 nM), using Lipofectamine 2000 (Invitrogen), as per the manufacturer's instructions. The oligomer–lipofectamine complexes were added to the cells. After 8 h, the transfection medium was changed to fresh culture medium and the cells were then assayed for levels of $VPAC_1$-receptor protein and cell proliferation. A nonsilencing RNA duplex (Ambion) was used as a control. The effective sequences were 5′-GGA GGA GUG UAG CUA UGU G-3′ for duplex 1, and 5′-CAC AUA GUC ACA CUC CUC C-3′ for duplex 2.

2.11. Animals and preparation of A498 cell xenografts

Athymic nude (nu/nu) 4 week-old male mice were purchased from Harlan Iberica Laboratory (Barcelona, Spain) and maintained under specific pathogen-free conditions. We obtained approval from the Institutional Animal Care and Use Committee of Alcala University. Mice were housed in Plexiglas cages (four/cage), kept at 20 °C with a 12-h light/dark cycle, and fed autoclaved standard chow and water *ad libitum*. A498 cells were preincubated in the absence or presence of 3 μM VIP antagonist, JV-1-53 for 10 min, and then they were incubated in the absence or presence of 1 μM VIP for 1 h. Afterwards, cells were washed with PBS, detached with 25% trypsin/0.2% EDTA, centrifuged at 400×*g*, and resuspended in fresh medium. Cell suspension was mixed with Matrigel (BD Bioscience) synthetic basement membrane (1:1, v/v) and then injected under the skin in the abdomen (5×10^6 cells/mouse). Six animals were used per group. Tumors were harvested after sacrifice at 3 weeks of subcutaneous cell injection.

2.12. Data analysis

Quantification of band densities was performed by using the Quantified One Program (Bio-Rad). Data shown in the figures are representative of at least three different experiments. The results are expressed as the mean ± SEM and were statistically treated following a Bonferroni's test for multiple comparisons after one or two-way analysis of variance (ANOVA). The level of significance was set at $P<0.05$.

3. Results

3.1. VIP mRNA and peptide levels

Non-tumor (HK2) and tumor (A498) human proximal tubular epithelial cell lines were investigated for the expression of mRNA for VIP by semiquantitative RT-PCR as well as for VIP protein by EIA procedures. Semiquantitative RT-PCR analysis of VIP mRNA expression yielded a product of the expected size for both cell lines. The expression of mRNA for β-actin was used as internal control. Fig. 1 shows that both mRNA for VIP and peptide VIP were overexpressed in tumor cells. In the second case, the increase of peptide levels in A498 cells was seen in both cytosolic fraction and extracellular medium which indicates that tumor cells are more active than non-tumor cells in the production and secretion of VIP.

3.2. Effect of exogenous VIP on intracellular VIP levels and VIP secretion

In order to investigate whether VIP increases its own expression and secretion, VIP levels were measured by EIA in both intracellular and extracellular media from HK2 and A498 cells that had been incubated in the absence or presence of 0.1 μM VIP for different times. The results showed that cytosolic VIP levels were maximal at 2 h in both cell lines (Fig. 2, upper panels). These increases in cytosolic VIP levels were conceivably a result of an increased expression of the neuropeptide. In order to determine whether these elevated levels in the intracellular compartment were accompanied by an enhancement of VIP secretion, EIA was performed in the secreted medium. As shown in Fig. 2 (lower panels), the results indicated that extracellular VIP reached maximal levels at 16 h and 8 h in HK2 and A498 cells, respectively.

3.3. Expression of VIP receptors

The expression of VIP receptors was determined in both non-tumor HK2 and tumor A498 cells by means of immunocytochemistry using specifics antibodies. Both cell lines expressed $VPAC_1$, $VPAC_2$ and PAC_1 receptors, whereas FPRL-1 receptor was only expressed in HK2 cells (Fig. 3). $VPAC_1$ was highly expressed at the nuclear level in both cell lines, whereas $VPAC_2$ and PAC_1 receptors were expressed in both cells lines but they

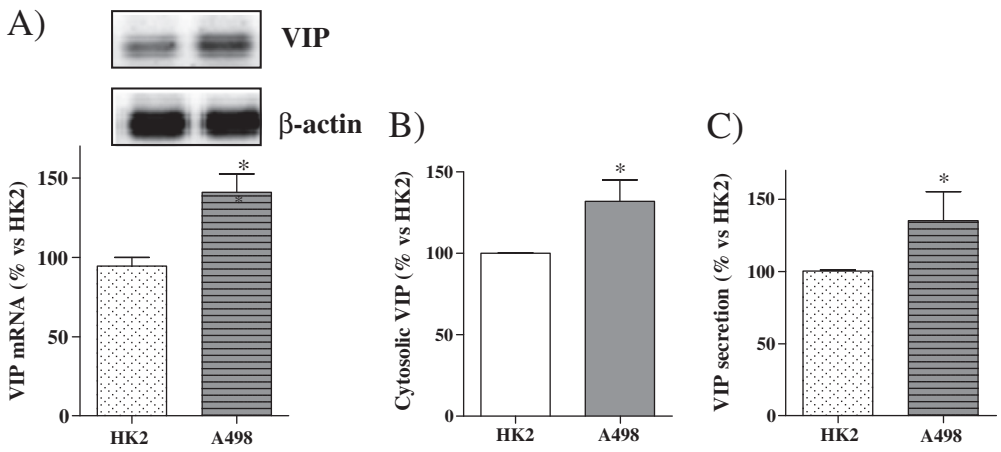

Fig. 1. A: Expression of VIP mRNA in HK2 and A498 cells as studied by semiquantitative RT-PCR. PCRs yielded products of the expected size (101 bp). All preparations were normalized according to the expression of β-actin mRNA. B: Expression of VIP (cytosolic fraction) as measured by ELISA. C: Expression of VIP (extracellular medium). Data are the means ± S.E. of at least three experiments. *P<0.05.

did not show any specific location. With regard to FPRL-1, it was found to be expressed in the plasma membrane fraction of HK2 cells.

3.4. Coupling of VIP receptors to the cAMP system

The cAMP molecule is recognized as the major second messenger of the VIP-receptor system. The ability of VIP to stimulate cAMP production in both human proximal tubular epithelial renal cells studied proved the functionality of VIP receptors (Fig. 4A). The response to VIP was dose-dependent and qualitatively as well as quantitatively similar in HK2 and A498 cells. Forskolin, a diterpene that interacts with adenylyl cyclase in a direct way, also increased cAMP levels by a dose-dependent mechanism in both cell lines (Fig. 4B).

3.5. Effect of VIP on cell proliferation

The study of mitochondrial functionality indicated that VIP did not modify cell viability in any cell line studied. This finding indicated that VIP did not exert cytotoxic effects in renal cells. Indeed, treatment with VIP did not induce apoptosis or necrosis as indicated by flow-cytometry analysis of human proximal tubular epithelial cell lines labeled with propidium iodide and annexin V (results not shown).

The effect of VIP on the proliferation of HK2 and A498 cells was estimated by means of [^{3}H]-thymidine and BrdU incorporation assays. Treatment for 24 h with 0.1 μM VIP ([^{3}H]-thymidine assays) (Fig. 5A) or increasing VIP concentrations (BrdU assays) (Fig. 5B) resulted in a significant decrease of cell proliferation in A498 cells, as compared with untreated controls. However, VIP treatment did not modify cell

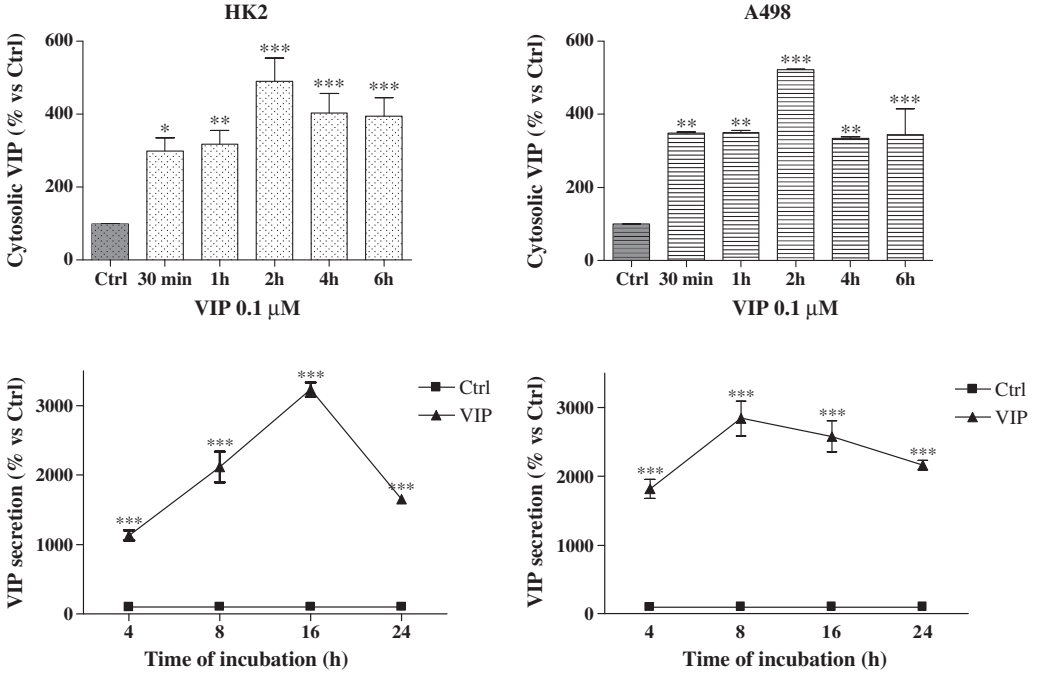

Fig. 2. Effect of VIP on its own expression and secretion in human renal cells. Both renal cell lines were incubated in the absence (Ctrl) or presence of 0.1 μM VIP for increasing times. Upper panels: VIP expression was estimated by measuring the cytosolic levels of the neuropeptide by EIA. Lower panels: in order to evaluate VIP secretion, the cells were incubated in the absence or presence of 0.1 μM VIP for 30 min and then the medium was replaced and the incubation continued for the indicated times; finally, the levels of VIP were measured in extracellular medium by EIA. Data are the means ± S.E. of at least five experiments. *P<0.05, **P<0.01, ***P<0.001.

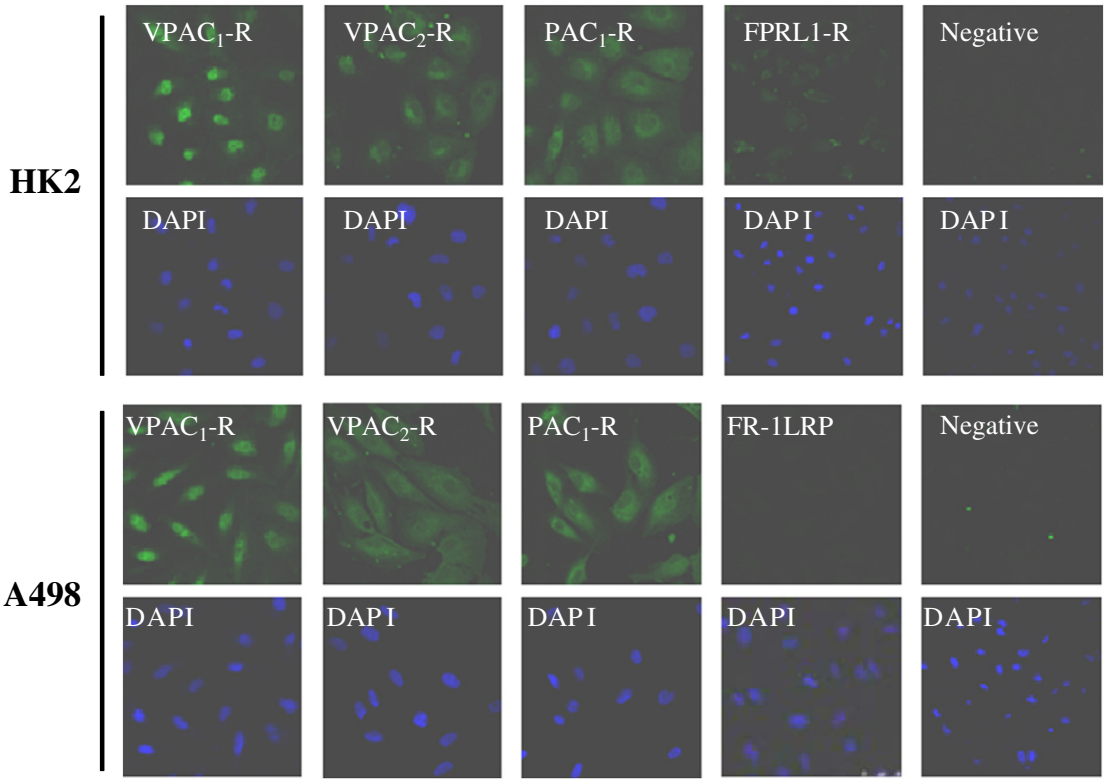

Fig. 3. Inmunodetection of VIP receptors ($VPAC_1$-R, $VPAC_2$-R, PAC_1-R and FPRL-1-R) was carried out by confocal immunofluorescence microscopy as described under Section 2.4. Representative images from four experiments are shown.

proliferation in HK2 cells. PACAP38, a VIP-related peptide that also acts through $VPAC_{1/2}$ receptors (in addition to PAC_1 receptors), showed also a growth inhibitory pattern upon A498 cells as observed with BrdU

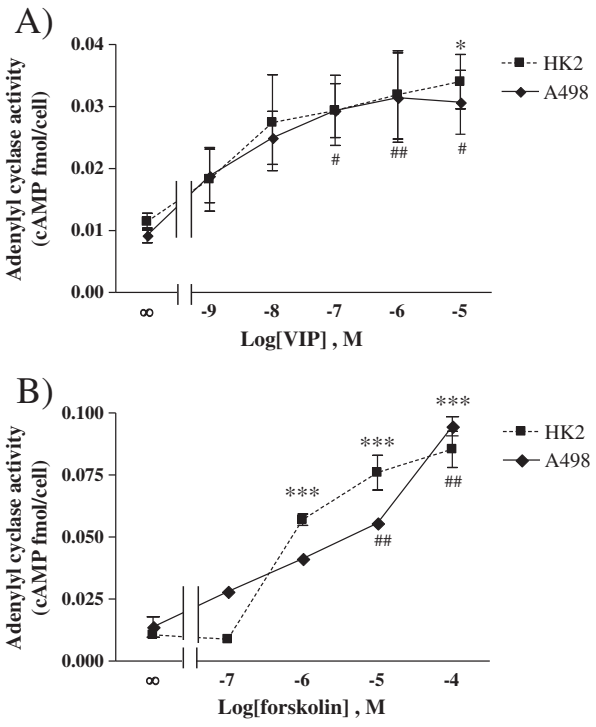

Fig. 4. Functionality of VIP receptors. Stimulation of cAMP production by increasing concentrations of VIP (A) and forskolin (B). Data are the means ± S.E. of five independent experiments. $^{*}P<0.05$ compared with basal level in HK2 cells, and $^{\#}P<0.05$, $^{\#\#}P<0.01$ compared with basal levels in A498 cells.

experiments (data not shown). Results with VIP were confirmed by the analysis of the expression of PCNA (Fig. 5C): treatment with 0.1 μM VIP for 1 h resulted in a decrease of about 25% of PCNA levels after 1–2 h in A498 cells but HK2 were unaffected. Thus, VIP appears to be involved in antiproliferative mechanisms in renal cell carcinomas but not in benign renal tissues.

3.6. Effect of VIP on STAT3 expression

We also determined whether VIP regulated STAT3 expression at both mRNA and protein levels. For this purpose, HK2 and A498 cells were treated with 0.1 μM VIP for different times. Semiquantitative RT-PCR for STAT3 (Fig. 6A) and measurement of pSTAT3/STAT3 protein levels by means of Western blot analysis (Fig. 6B) followed by densitometry were then carried out. Renal cell carcinoma A498 cells, but not HK2 cells, showed a decrease of STAT3 mRNA expression. In addition, VIP decreased phosphorylation and activation of STAT3 in tumor cells, but not in HK2 cells. These findings on effects of VIP on STAT3 expression correlate with the observations on VIP regulation of cell proliferation in renal cell carcinoma.

3.7. Involvement of $VPAC_1$-receptor on renal cell carcinoma proliferation

In order to characterize $VPAC_1$ receptor (the main VIP receptor) involvement in proliferation of renal tumor cells, A498 cells were treated with 0.3 μM VIP antagonist, JV-1-53 for 24 h in the absence or presence of 0.1 μM VIP. After a 30 min incubation with 10 μM BrdU, measurement of cell proliferation by BrdU incorporation assay showed that the inhibitory effect was blocked by JV-1-53 (Fig. 7A), suggesting the involvement of $VPAC_1$ receptor. To further establish the participation of $VPAC_1$-receptors in renal cell carcinoma proliferation, A498 cells were transfected with $VPAC_1$-receptor siRNA. The study of the expression of the receptor by Western blot showed a drastic decrease (by 95%) of $VPAC_1$-receptor levels (Fig. 7B). Moreover, transfected A498 cells presented an increased

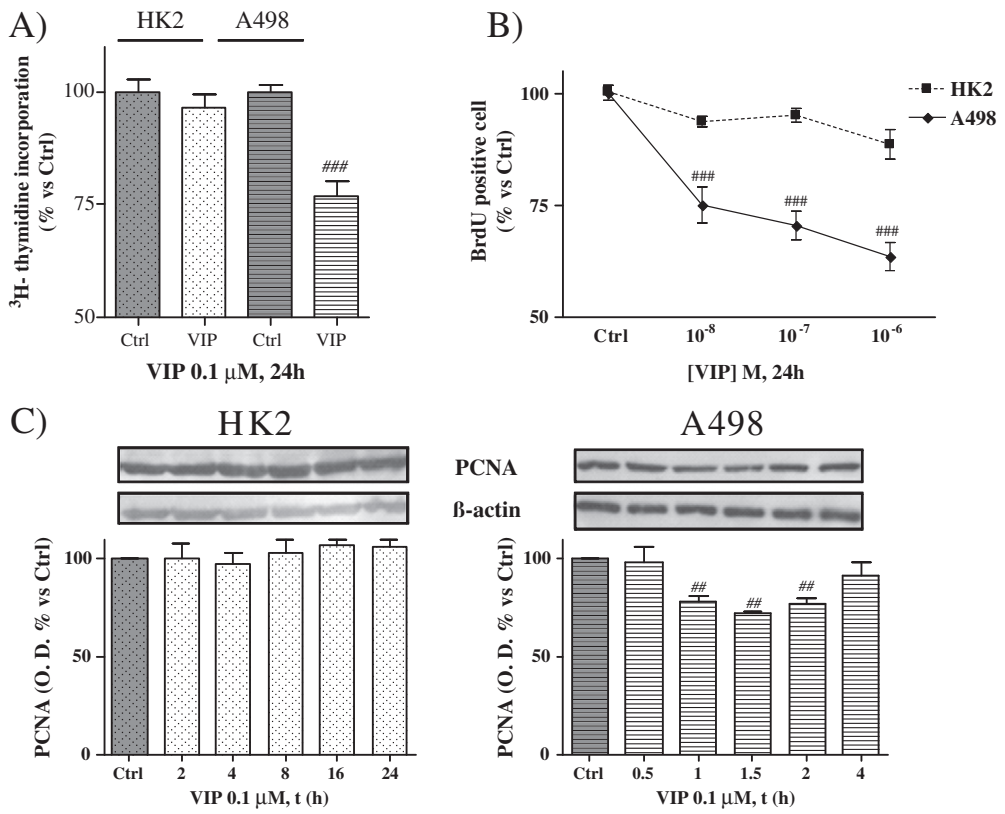

Fig. 5. VIP effect on cell proliferation (A and B) and PCNA protein levels (C). A: [^{3}H]-thymidine incorporation; HK2 and A498 cells were incubated in the absence or presence of 0.1 μM VIP and [^{3}H]-thymidine (0.4 μCi/ml). B: BrdU incorporation; cells were incubated for 24 h in the absence or presence of increasing doses of VIP and 10 μM BrdU. C) Western-blot of PCNA; PCNA levels were determined in both cell lines after treatment with 0.1 μM VIP at different times. A representative experiment is shown. The bar represent the means ± S.E. of six experiments. $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ compared with control.

expression of pSTAT3/STAT3 ratio (Fig. 7B) which supports the involvement of $VPAC_1$ receptor in activation of STAT3 protein activation. Finally, the effect of $VPAC_1$ receptors on tumor cell proliferation was measured by means of BrdU incorporation assays in transfected A498 cells. The results showed a significant increase in the proliferation extent of transfected cells as compared to negative, non-transfected cells (Fig. 7C).

3.8. Signal transduction pathways involved in VIP effect on cell proliferation

To explore whether the effect of VIP on A498 cell proliferation is related with EPAC-mediated pathways, cells were incubated for 24 h with 0.1 μM VIP, 300 μM 8-pCPT-2′-O-Me-cAMP or 0.1 μM forskolin (Fig. 8A). Thereafter, the cells were incubated with 10 μM BrdU and cell proliferation was measured by BrdU incorporation assay. The involvement of the cAMP/EPAC pathway was confirmed by the observation of the inhibitory effects of forskolin and 8-pCPT-2′-O-Me-cAMP upon cell proliferation.

To evaluate the specificity of the signaling pathways that could be involved in the VIP effect on the proliferation of renal carcinoma cells, A498 cells were cultured in the absence or presence of 0.1 μM VIP and different protein kinase inhibitors: PKA inhibitor H89, MEK1/2 inhibitor PD98059, or PI3-K inhibitor wortmannin. Again, the cells were incubated with 10 μM BrdU and cell proliferation was measured by BrdU incorporation assay. As shown in Fig. 8B, cell treatment with 0.1 μM wortmannin fully abolished the inhibitory effect of VIP upon proliferation of A498 cells, whereas 10 μM PD98059 had no effect and 10 μM H89 resulted in decreased cell proliferation. These results support that PI3-K plays a role in mediating the inhibitory effects of VIP on proliferation of A498 cells.

3.9. VIP prevention of tumorigenesis by A498 cells in xenografted athymic mice

The efficacy of VIP on the prevention of tumor progression was also studied in vivo. Using xenografted athymic mouse, we observed that growth of the tumor mass was initiated as soon as 2 days after the inoculation of tumoral A498 cells and all groups developed tumors. Also, cells treated with the neuropeptide (1 μM VIP for 1 h) presented a significant decrease (30%) in their ability to develop tumor growth as shown from the beginning of development. The inhibitory effect of VIP upon developing tumors was reverted by using A498 cells that had been preincubated with the antagonist JV-1-53 (3 μM VIP antagonist for 10 min), which indicates the involvement of $VPAC_1$-receptor in the antitumoral effect of the neuropeptide (Fig. 9).

4. Discussion

In this study, we demonstrate for the first time that VIP inhibits the proliferation of A498 human renal carcinoma cells both in vitro and in vivo. The use of different experimental approaches to generate our findings as well as the observations of the role of EPAC/STAT3 signal transduction system in VIP activity support this conclusion. These results together with the detection of the expression of VIP and its receptors in renal tumor cells provide molecular basis for potential development of new therapies for renal cancer.

Present results are in apparent conflict with previous reports on the stimulatory effect of VIP on cell proliferation and related events in normal and tumor cells [26–29]. Thus, we have demonstrated that VIP induces promotion and progression of human prostate carcinoma and potentiates the production of key proteins involved in development of metastasis

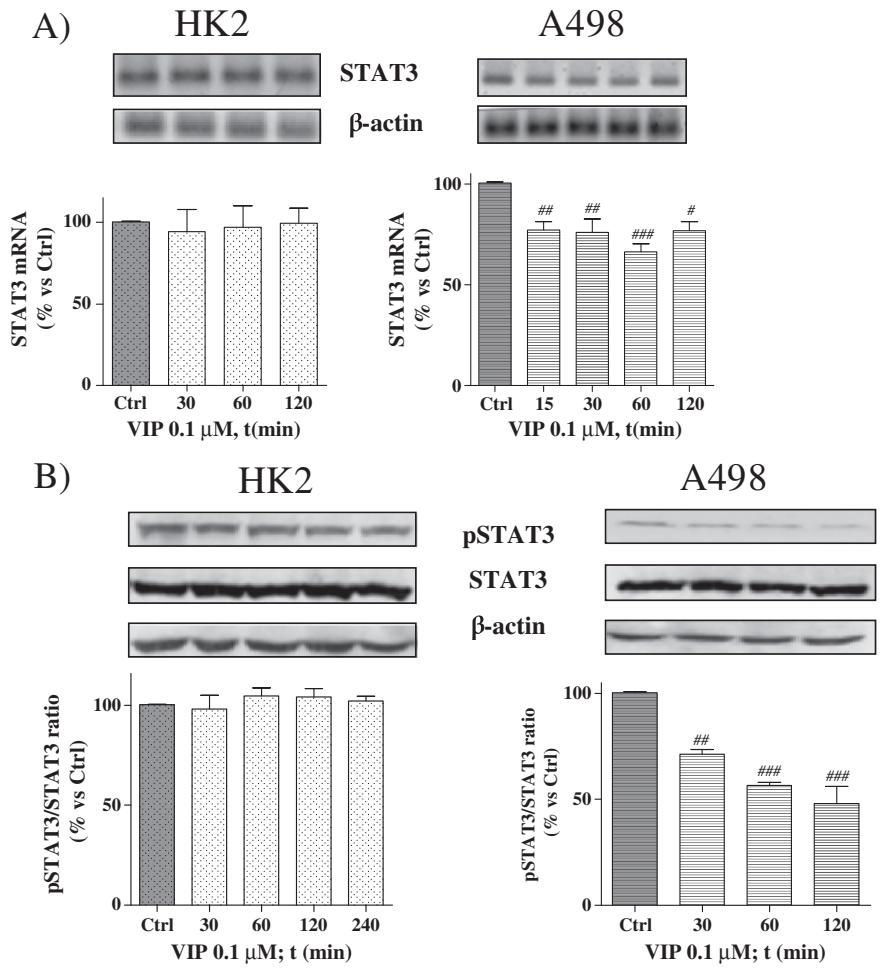

Fig. 6. VIP effect on STAT3 mRNA expression (A) and pSTAT3/STAT3 ratio protein levels (B). A: STAT3 mRNA levels; cells were treated with 0.1 μM VIP and, after incubation, total RNA was assayed for STAT3 and β-actin levels by RT-PCR and densitometry. B: Western-blot of pSTAT3 and STAT3; levels of pSTAT3 and STAT3 were determined in both cells after treatment with 0.1 μM VIP at different times. A representative experiment is shown. The bar diagrams represent the means ± SE of six experiments. $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ compared with control.

[26–29]. Similarly, VIP promotes cell growth of other tumors including human breast carcinoma [30–32]. These responses are the result of interaction of VIP with its receptors, mainly $VPAC_1$ receptors, which are coupled through G_s proteins to adenylyl cyclase activation. The resulting cAMP increase leads to PKA activation, phosphorylation of protein substrates, and potentiation of oncogene expression [16,33]. In addition to the activation of cAMP/PKA as its major signal transduction mechanism, VIP may act also through other systems such as PI3-K and extracellular signal-regulated kinases 1 and 2 (ERK 1/2) [26,33]. The observations of the present study on the antiproliferative effect of VIP on RCC are similar to those on other tumors that included, among others, neuroblastoma [21] and hepatocellular carcinoma [22]. The inhibitory effect of VIP

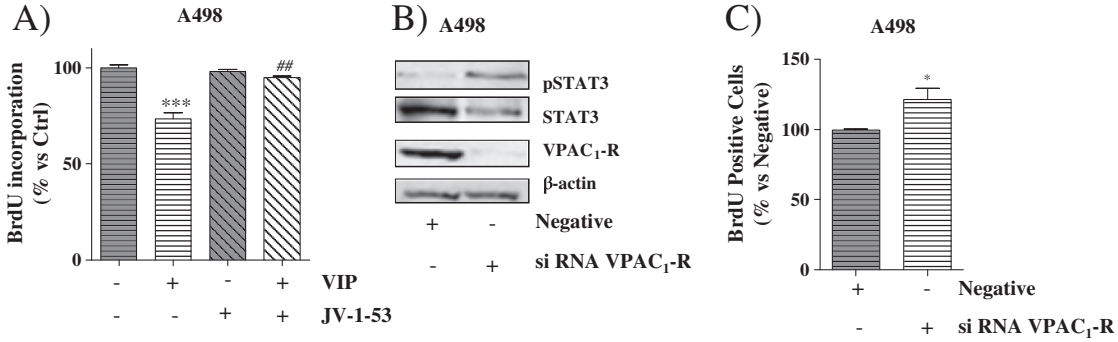

Fig. 7. Involvement of $VPAC_1$ receptor in the proliferation of renal cell carcinoma cells. A: A498 cells were incubated for 24 h with the specific antagonist JV-1-53 in the absence or presence of 0.1 μM VIP. Then cells were incubated with 10 μM BrdU for 30 min and cell proliferation were measured by BrdU incorporation assay. A498 cells were transfected with $VPAC_1$-receptor siRNA; thereafter, the levels of VPAC1 receptor were estimated by Western blotting in parallel to non-transfected cells. Levels of pSTAT3/STAT3 were determined in both transfected and non-transfected cells to show $VPAC_1$-receptor role on STAT3-mediated cell proliferation. C: Effect of transfection of A498 cells with $VPAC_1$-receptor siRNA on cell proliferation as measured by BrdU incorporation assay. A representative experiment is shown. The bar diagrams represent the means ± S.E. of three experiments. $^{*}P<0.05$, $^{***}P<0.001$ compared with control or scramble, and $^{\#\#}P<0.01$ compared with VIP.

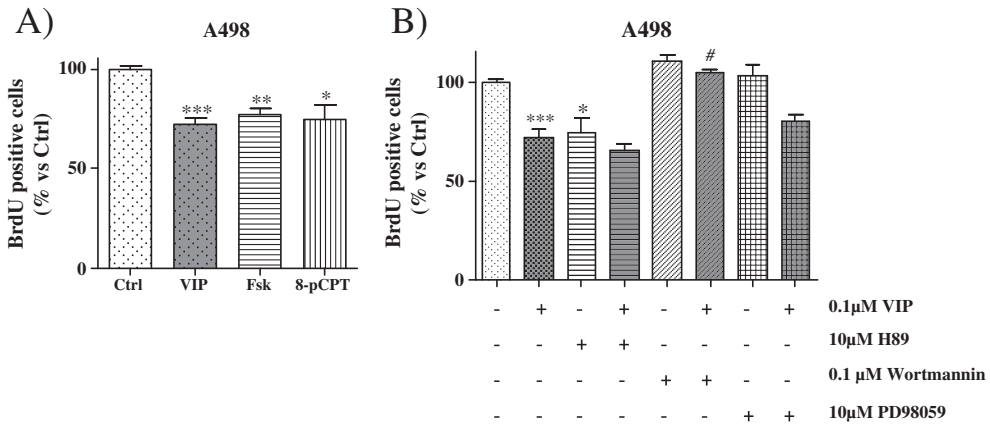

Fig. 8. Signal transduction pathways involved in VIP effect on cell proliferation as measured by BrdU incorporation. A: Effects of forskolin and cAMP agonist on cell proliferation. A498 cells were incubated for 24 h with 0.1 μM VIP (24 h), 0.1 μM forskolin or 300 μM 8-pCPT-2′-O-Me-cAMP followed by incubation with 10 μM BrdU for 30 min. B: Effects of protein kinase inhibitors on cell proliferation. Cells were pretreated with H89 (15 min), wortmannin (30 min) or PD98059 (1 h) and then incubated in the absence or presence of 0.1 μM VIP (24 h) followed by 10 μM BrdU (30 min). A representative experiment is shown. The bar diagrams represent the means ± S.E. of four experiments. *P<0.05, **P<0.01, ***P<0.001 compared with control, and #P<0.05 compared with VIP.

upon cell growth in these cancer cell systems conceivably derives from alternative signaling models as EPAC/STAT3 studied here. Thus, the occurrence of different receptor molecules for VIP together with the complex array of intracellular signaling pathways that can be activated after VIP-receptor activation explains the observation of apparent discrepancies such as the opposite effects of the neuropeptide on cancer cell proliferation depending on the affected organ.

We used non-tumoral HK2 and tumoral A498 renal cells. Both human cell lines expressed VIP, as assessed by mRNA and peptide determinations, and VIP secretion towards extracellular medium was higher in non-tumoral cells. These findings together with the characterization of $VPAC_1$, $VPAC_2$ and PAC_1 receptors in both cell lines suggest autocrine and/or paracrine actions of this neuropeptide in RCC as observed in other systems [26–33]. Immunolocalization of VIP receptors allowed us to detect FPRL-1 receptors in HK2, but not in A498 cells. This GPCR subtype has been characterized recently as an inhibitor of cAMP signaling [17]. In other context, the finding of different subtypes of VIP receptors not only in the plasma membranes, but also in intracellular compartments is not surprising since it is a known feature for an increasing number of peptide receptors including those for VIP [32].

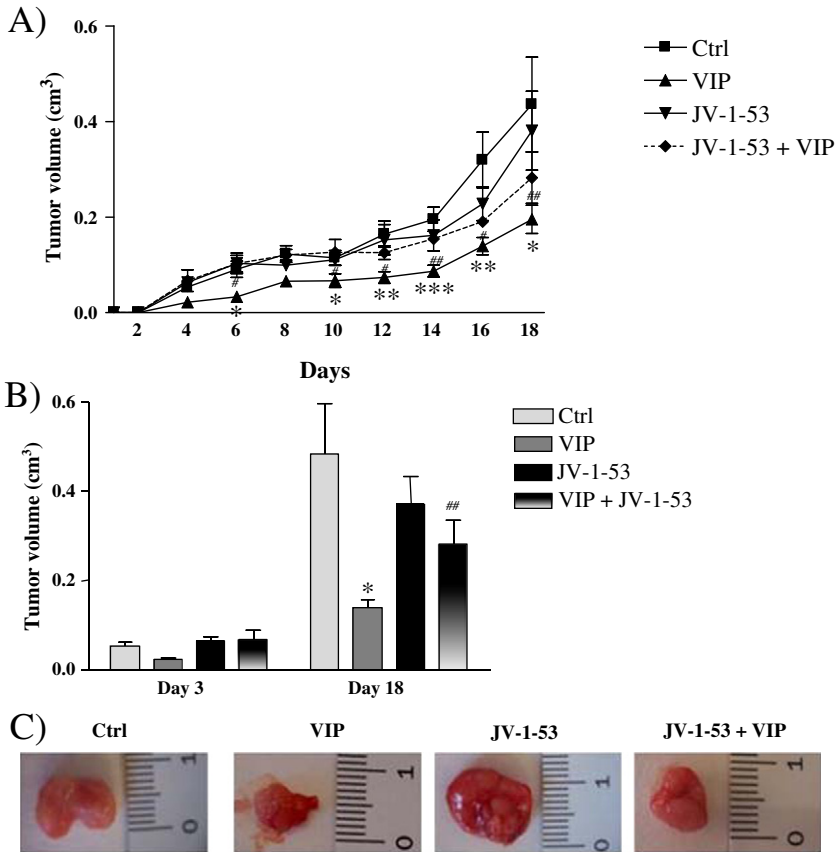

Fig. 9. Effect of VIP on tumor growth after subcutaneous injection of Matrigel-A498 cells in nude mice. Cells were incubated in the absence or presence of 3 μM VIP antagonist, JV-1-53 (10 min) and/or 1 μM VIP, mixed with Matrigel, and subcutaneously injected in nude mice. After sacrifice at 18 days, tumors were removed for volume measurement. A representative experiment is shown. The bar diagrams represent the means ± S.E. *P<0.05, **P<0.01, ***P<0.001 compared with control, and #P<0.05 compared with VIP.

VIP effects were studied by means of cell incubation with the peptide, 0.1 μM VIP for 24 h. In agreement with previous results [28], VIP had no toxic effects on cell viability that could mask the observed responses. The study of mitochondrial functionality as well as cell cycle analysis by flow cytometry excluded the possibility of apoptosis or necrosis due to VIP treatment. Interestingly, the neuropeptide inhibited the proliferation of tumor cells but it did not affect non-tumoral cell growth as indicated by assays on [^{3}H]-thymidine and BrdU incorporation as well as measurement of PCNA levels. These results are in close agreement with our parallel observations on STAT3 expression and activation. STAT3 appears to be involved in oncogenic transformation through gene transcription in antiapoptotic pathways, as shown in various cancer types including renal cell cancer [11–13]. Down-regulation by VIP of STAT3 expression in malignant A498 but not in non-tumoral HK2 renal cells and inhibition by VIP of STAT3 activation (by phosphorylation) in A498 cells support the view that the neuropeptide exerts its antiproliferative effects in tumor renal cells through the inhibition of both STAT3 and pSTAT3 levels.

Since STATs are a major point of convergence for different signaling systems that are activated in tumor cells [7], it is mandatory to define upstream signals that connect with VIP in renal cancer cells. First of all, the major role of $VPAC_1$ receptor in the antiproliferative activity of VIP on A498 cells emerges from our observation with the VIP antagonist JV-1-53, a GHRH analog that abolished this VIP effect and restored cell proliferation to basal levels. JV-1-53 is a VIP-receptor antagonist previously tested in other tumors [24,25,34]. JV-1-53 shows an almost negligible affinity for GHRH receptors, similar to that of VIP. JV-1-53 structure is: [Ac-His^1,D-Phe^2,Phe(4-Cl)^6,Lys^{15},Arg^{16},Lys^{20},Tyr^{22},Nle^{27},D-Arg^{28},Har^{29}]hGHRH(1–29)NH_2. JV-1-53 contains a D-Phe^2 substituent instead of D-Arg^2, because this substitution produces a predominantly VIP antagonistic property on incorporation into GHRH analogs [24,25]. VIP antagonist JV-1-53 has several additional substitutions intended to increase the binding to VIP receptors and decrease its affinity to GHRH receptors such as the incorporation of Lys^{15} (also found in native VIP) and Arg^{16} residues [24,25]. Tyr^{22} increases the binding to $VPAC_2$ receptors and confers enhanced $VPAC_2$-receptor antagonistic activity to the analog in addition to its potent $VPAC_1$-receptor inhibitory activity. JV-1-53 also contains Lys^{20} substitution, characteristic of native VIP. As in other GHRH analogs, Lys^{20} drastically reduces GH-releasing activity and decreases the affinity to GHRH receptors. JV-1-53 is not able to inhibit GHRH-stimulated GH response, and its GHRH-receptor binding affinity is decreased as compared with GHRH antagonists JV-1-36 and JV-1-38 [25]. In previous studies, GHRH antagonists MZ-4-71 ([Ibu-Tyr^1,D-Arg^2,Phe(4-Cl^6),Abu^{15},Nle^{27}]hGHRH(1–28)Agm) and JV-1-38 [PhAc-Tyr^1,D-Arg^2,Phe(4-Cl)^6,Har^9,Tyr(Me)^{10},Abu^{15},Nle^{27},D-Arg^{28},Har^{29}]hGHRH(1–29)NH_2) inhibited in vivo growth of CAKI-1-RCC [35,36]. The antagonist JV-1-53 showed high binding affinity to VIP receptors on CAKI-1 cancer cells [36]. Since the structure of JV-1-53 is based on GHRH(1–29) and related to those of MZ-4-71 and JV-1-38, the findings reported in this paper and contrasted with the results cited above [35,36] demonstrate a high specificity of both the analogs and the receptors for GHRH and VIP.

The evidence for antiproliferative effects of VIP was reinforced by the silencing of $VPAC_1$ receptor with siRNA which resulted in an almost complete absence of $VPAC_1$ receptors in renal tumor cells. In addition, we also observed increases in both cell proliferation and expression of pSTAT3/STAT3 ratio in transfected cells. Thus, antiproliferative effects of VIP in A498 cells are initiated by $VPAC_1$ receptors and involve activation of STAT3. Silencing of VIP receptors by siRNA transfection has been carried out in a limited number of studies including those on human prostate epithelial cells [29], breast cancer cells [37] and embryonic kidney cells [38].

Our experiments show that VIP dose-dependently stimulates adenylyl cyclase activity in both HK2 and A498 cells. The same pattern was observed by direct activation of the enzyme with forskolin. Moreover, VIP, forskolin, and the EPAC agonist 8-pCPT-2′-O-Me-cAMP inhibited proliferation of A498 cells, as measured by BrdU incorporation assays. These results support that the antiproliferative effects of VIP could be mediated by a cAMP-dependent mechanism in a similar manner to that seen in other tumors such as neuroblastoma [21] and hepatocellular carcinoma [22]. In renal tumor cells, the cAMP signal is conceivably connected with STAT3 through PI3-K transduction pathway as suggested by the abrogating effect of the PI3-K inhibitor wortmannin on antiproliferative activity of VIP. In small cell lung carcinoma [39,40], VIP inhibited cell proliferation through the cAMP/PKA pathway whereas the effect of this neuropeptide was cAMP independent in C6 glioma cells [41]. It is important to note that the observations in vitro on the growth inhibitory effects of VIP were confirmed in vivo by means of xenografting of A498 cells (treated with VIP and/or the specific antagonist JV-1-53) in athymic nude mice. The observed VIP effect in developing tumors after subcutaneous injection of VIP-treated A498 cells was a reduction of tumoral mass of about 30%. The involvement of $VPAC_1$-receptor in this response was indicated by the reversion of the VIP effect with cell incubation in the presence of the specific antagonist JV-1-53.

In conclusion, our results support that the VIP-receptor system may be considered one of the components involved in decreased proliferation of renal cancer cells, as assessed by the study of VIP treatment in the incorporation of [^{3}H]-thymidine and BrdU, PCNA expression and STAT3 expression and activation in tumoral A498 cells. Cell growth-inhibitory response to VIP involves $VPAC_1$-receptor participation and appears to be related to the EPAC/PI3-K signaling systems. Moreover, in vivo results with xenografted athymic nude mice confirm the growth inhibitory effect of VIP and the involvement of $VPAC_1$ receptor, which represents the first description of an antitumoral effect of VIP in an animal model. Present data must be added to those obtained on the protective role of the VIP-related peptide PACAP in kidney cells as well as in multiple myeloma and other kidney diseases [19,20]. Our findings suggest the merit of further molecular studies on signaling pathways and transcriptional factors responsible for tumor growth suppression aimed at development of effective VIP-related molecules for potential therapy of renal cell carcinoma.

Acknowledgements

This work was financially supported by the Ministerio de Ciencia e Innovación (grant SAF2007-63794) and the Comunidad Autónoma de Castilla la Mancha (grant PII 1/09-0061-3802).

References

[1] H.T. Cohen, F.J. Mc Goven, Renal-cell carcinoma, N. Engl. J. Med. 353 (2005) 2477–2490.
[2] B. Ljungberg, S.C. Campbell, H.Y. Cho, D. Jackmin, J.E. Lee, S. Weikert, L.A. Kiemeney, The epidemiology of renal cell carcinoma, Eur. Urol. 60 (2011) 615–621.
[3] F. Algaba, H. Akaza, A. López-Beltrán, G. Martignoni, H. Moch, R. Montironi, V. Reuter, Current pathology keys of renal cell carcinoma, Eur. Urol. 60 (2011) 634–643.
[4] Z. Kirkali, M. Lekili, Renal cell carcinoma: new prognostic factors? Curr. Opin. Urol. 13 (2003) 433–438.
[5] R.J. Motzer, R.M. Bukowski, Targeted therapy for metastatic renal cell carcinoma, J. Clin. Oncol. 24 (2006) 5601–5608.
[6] C. Eichelberg, K. Junker, B. Ljungberg, H. Moch, Diagnostic and prognostic molecular markers for renal cell carcinoma: a critical appraisal of the current state of research and clinical applicability, Eur. Urol. 55 (2009) 851–863.
[7] H. Yu, R. Jove, The STATs of cancer — new molecular targets come of age, Nat. Rev. Cancer 4 (2004) 97–105.
[8] H. Yu, D. Pardoll, R. Jove, STATs in cancer, inflammation and immunity: a leading role for STAT3, Nat. Rev. Cancer 9 (2009) 798–809.
[9] J. Yang, G.R. Stark, Roles of unphosphorylated STATs in signaling, Cell Res. 18 (2008) 443–451.
[10] D.R. Hodge, E.M. Hurt, W.L. Farrar, The role of IL-6 and STAT3 in inflammation and cancer, Eur. J. Cancer 41 (2005) 2502–2512.
[11] J. Wang, C. Ouyang, X. Chen, B. Fu, Y. Lu, Q. Hong, STAT3 inhibits apoptosis of human renal tubular epithelial cells induced by ATP depletion/recovery, Nephron Exp. Nephrol. 108 (2008) e11–e18.
[12] B.B. Agarwal, A.B. Kunnumakkara, K.B. Harikumar, S.R. Gupta, S.T. Tharakan, C. Koka, S. Dey, B. Sung, Signal transducer and activator of transcription-3, inflammation and cancer, Ann. N. Y. Acad. Sci. 1171 (2009) 59–76.
[13] A. Horiguchi, M. Oya, T. Shimada, A. Uchida, K. Marumo, M. Murai, Activation of signal transducer and activator of transcription 3 in renal cell carcinoma: a

study of incidence and its association with pathological features and clinical outcome, J. Urol. 168 (2002) 762–765.
[14] A. Masuda, T. Kamai, H. Abe, K. Arai, K. Yoshida, Is STAT3 and/or p53 mRNA expression a prognostic marker for renal cell carcinoma? Biomed. Res. 30 (2009) 171–176.
[15] M. Laburthe, A. Couvineau, J.C. Marie, VPAC receptors for VIP and PACAP, Recept. Channels 8 (2002) 131–153.
[16] M. Laburthe, A. Couvineau, V. Tan, Class II G-protein coupled receptors for VIP and PACAP: structure, models of activation and pharmacology, Peptides 28 (2007) 1631–1639.
[17] N. El Zein, B. Badran, E. Sariban, VIP differentially activates beta2 integrins, CR1, and matrix metalloproteinase-9 in human monocytes through cAMP/PKA, EPAC, and PI-3K signaling pathways via VIP receptor type I and FPRL1, J. Leukoc. Biol. 72 (2008) 972–981.
[18] G. Borland, B.O. Smith, S.J. Yarwood, EPAC proteins transduce diverse cellular actions of cAMP, Br. J. Pharmacol. 158 (2009) 70–86.
[19] G. Horvath, R. Brubel, K. Kovacs, D. Reglodi, B. Opper, A. Ferencz, P. Szakaly, E. Laszlo, L. Hau, P. Kiss, A. Tamas, B. Racz, Effects of PACAP on oxidative stress-induced cell death in rat kidney and human hepatocyte cells, J. Mol. Neurosci. 484 (2010) 148–152.
[20] Li, J.L. Maderdrut, J.J.L. Lertora, A. Arimura, V. Batuman, Renoprotection by pituitary adenylate cyclase-activating polypeptide in multiple myeloma and other kidney diseases, Regul. Pept. 145 (2008) 24–32.
[21] J.M. Muller, M. Philipe, L. Chevrier, C. Heraud, C. Alleaume, C. Chadeneau, The VIP-receptor system in neuroblastoma cells, Regul. Pept. 137 (2006) 34–41.
[22] A. Absood, B. Hu, N. Bassily, L. Colletti, VIP inhibits human HepG2 cell proliferation in vitro, Regul. Pept. 146 (2008) 285–292.
[23] A.G. Gilman, A protein binding assay for adenosine 3′–5′-cyclic monophosphate, Proc. Natl. Acad. Sci. U.S.A. 67 (1970) 305–312.
[24] J.L. Varga, A.V. Schally, V.J. Csernus, M. Zarandi, G. Halmos, K. Groot, Z. Rekasi, Synthesis and biological evaluation of antagonists of growth hormone-releasing hormone with high and protracted in vivo activities, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 692–697.
[25] Z. Rekasi, J.L. Varga, A.V. Schally, G. Halmos, K. Groot, T. Czompoly, Antagonistic actions of analogs related to growth hormone-releasing hormone (GHRH) on receptors for GHRH and vasoactive intestinal peptide on rat pituitary and pineal cells in vitro, Proc. Natl. Acad. Sci. U.S.A. 97 (2000) 1218–1223.
[26] M.G. Juarranz, O. Bolaños, I. Gutiérrez-Cañas, E.A. Lerner, P. Robberecht, M.J. Carmena, J.C. Prieto, N. Rodríguez-Henche, Neuroendocrine differentiation of the LNCaP prostate cancer cell line maintains the expression and function of VIP and PACAP receptors, Cell. Signal. 13 (2001) 887–894.
[27] A.B. Fernández-Martínez, B. Collado, A.M. Bajo, M. Sánchez-Chapado, J.C. Prieto, M.J. Carmena, Vasoactive intestinal peptide induces cyclooxygenase-2 expression through nuclear factor-κB in human prostate cell lines. Differential time-dependent responses in cancer progression, Mol. Cell. Endocrinol. 270 (2007) 8–16.
[28] A.B. Fernández-Martinez, A.M. Bajo, M. Sánchez-Chapado, J.C. Prieto, M.J. Carmena, Vasoactive intestinal peptide behaves as a prometastatic factor in human prostate cancer cells, Prostate 63 (2009) 774–786.
[29] A.B. Fernández-Martínez, A.M. Bajo, M.I. Arenas, M. Sánchez-Chapado, J.C. Prieto, M.J. Carmena, Vasoactive intestinal peptide (VIP) induces malignant transformation of the human prostate epithelial cell line RWPE-1, Cancer Lett. 299 (2010) 11–21.
[30] A. Valdehita, M.J. Carmena, B. Collado, J.C. Prieto, A. Bajo, Vasoactive intestinal peptide (VIP) increases vascular endothelial growth factor (VEGF) expression and secretion in human breast cancer cells, Regul. Pept. 144 (2007) 101–108.
[31] A. Valdehita, A.M. Bajo, A.V. Schally, J.L. Varga, M.J. Carmena, J.C. Prieto, Vasoactive intestinal peptide (VIP) induces transactivation of EGFR and HER2 in human breast cancer cells, Mol. Cell. Endocrinol. 302 (2009) 341–348.
[32] A. Valdehita, A.M. Bajo, A.B. Fernández-Martínez, M.I. Arenas, E. Vacas, P. Valenzuela, A. Ruíz-Villaespesa, J.C. Prieto, M.J. Carmena, Nuclear localization of vasoactive intestinal peptide (VIP) receptors in human breast cancer, Peptides 31 (2010) 2035–2045.
[33] I. Gutiérrez-Cañas, M.G. Juarranz, B. Collado, N. Rodríguez-Henche, A. Chiloeches, J.C. Prieto, M.J. Carmena, Vasoactive intestinal peptide induces neuroendocrine differentiation in the LNCaP prostate cancer cell line through PKA, ERK, and PI3K, Prostate 63 (2005) 44–55.
[34] A. Jungwirth, A.V. Schally, J. Pinski, K. Groot, P. Armatis, G. Halmos, Growth hormone-releasing hormone antagonist MZ-4-71 inhibits in vivo proliferation of Caki-I renal adenocarcinoma, Proc. Natl. Acad. Sci. U.S.A. 94 (1997) 5810–5813.
[35] G. Halmos, A.V. Schally, J.L. Varga, A. Plonowski, Z. Rekasi, T. Czompoly, Human renal cell carcinoma expresses distinct binding sites for growth hormone-releasing hormone, Proc. Natl. Acad. Sci. U.S.A. 97 (2000) 10555–10560.
[36] B. Collado, M.G. Sánchez, I. Díaz-Laviada, J.C. Prieto, M.J. Carmena, Vasoactive intestinal peptide (VIP) induces c-fos expression in LNCaP prostate cancer cells through a mechanism that involves Ca^{2+} signalling. Implications in angiogenesis and neuroendocrine differentiation, Biochim. Biophys. Acta 1744 (2005) 224–233.
[37] A. Valdehita, M.J. Carmena, A.M. Bajo, J.C. Prieto, RNA interference-directed silencing of $VPAC_1$ receptor inhibits VIP effects on both EGFR and HER2 transactivation and VEGF secretion in human breast cancer cells, Mol. Cell. Endocrinol. 348 (2012) 241–246.
[38] F.F. Casanueva, J.P. Camiña, M.C. Carreira, Y. Pazos, J.L. Varga, A.V. Schally, Growth hormone-releasing hormone as an agonist of the ghrelin receptor GHS-R1a, Proc. Natl. Acad. Sci. U.S.A. 105 (2008) 20452–20457.
[39] K. Maruno, S.I. Said, VIP inhibits the proliferation of small-cell and nonsmall-cell lung carcinoma, Ann. N. Y. Acad. Sci. 805 (1996) 389–392.
[40] K. Maruno, A. Absood, S. Said, Vasoactive intestinal peptide inhibits human small-cell lung cancer proliferation in vitro and in vivo, Proc. Natl. Acad. Sci. U.S.A. 95 (1998) 14373–14378.
[41] P. Sokolowska, J.Z. Nowak, Effects of PACAP and VIP on cAMP-generating system and proliferation of C6 glioma cells, J. Mol. Neurosci. 36 (2008) 286–291.