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Céline Alleaume, Alain Eychène, Thomas Harnois, Nicolas Bourmeyster, Bruno Constantin, et al.. Vasoactive intestinal peptide-induced neurite remodeling in human neuroblastoma SH-SY5Y cells implicates the Cdc42 GTPase and is independent of Ras-ERK pathway.. *Experimental Cell Research*, 2004, 299 (2), pp.511-524. 10.1016/j.yexcr.2004.06.016 . hal-02880277

**HAL Id: hal-02880277**

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## Vasoactive intestinal peptide-induced neurite remodeling in human neuroblastoma SH-SY5Y cells implicates the Cdc42 GTPase and is independent of Ras-ERK pathway

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Received 5 April 2004, revised version received 15 June 2004

Available online 25 July 2004

### Abstract

Vasoactive intestinal peptide (VIP) is known to regulate proliferation or differentiation in normal and tumoral cells. SH-SY5Y is a differentiated cell subclone derived from the SK-N-SH human neuroblastoma cell line and possess all the components for an autocrine action of VIP. In the present study, we investigated the morphological changes and intracellular signaling pathways occurring upon VIP treatment of SH-SY5Y cells. VIP induced an early remodeling of cell projections: a branched neurite network spread out and prominent varicosities developed along neurites. Although activated by VIP, the Ras/ERK pathway was not required for the remodeling process. In contrast, pull-down experiments revealed a strong Cdc42 activation by VIP while expression of a dominant-negative Cdc42 prevented the VIP-induced neurite changes, suggesting an important role for this small GTPase in the process. These data provide the first evidence for a regulation of the activity of Rho family GTPases by VIP and bring new insights in the signaling pathways implicated in neurite remodeling process induced by VIP in neuroblastoma cells.

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**Keywords:** VIP; SH-SY5Y neuroblastoma cells; Neurite remodeling; Cdc42; ERK; Ras

### Introduction

The vasoactive intestinal peptide (VIP) belongs to the so-called secretin family of bioactive peptides, which also includes glucagon, a peptide having carboxy-terminal isoleucine/methionine (PHI/PHM) and pituitary adenylate

cyclase activating polypeptide (PACAP). These neuropeptides are widely distributed in peripheral and central nervous systems where they exert a large array of biological effects. Besides its action on exocrine secretions, smooth muscle relaxation and metabolism [1,2], and its trophic effect [3,4], VIP was also shown to regulate proliferation or differentiation in numerous cell lines, such as neuroblastoma, glioblastoma, pheochromocytoma, and a variety of epithelial tumors [5–13]. VIP acts through G-protein-coupled seven-transmembrane-domain receptors [14,15] and exerts many of its effects through activation of adenylate cyclase [14]. However, alternative signaling pathways have been

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proposed depending on cell type (Ref. [15] for review), including stimulation of phospholipase C, calcium mobilization, and stimulation of nitric oxide synthase.

Distinct subclones were derived from the human neuroblastoma cell line SK-N-SH originally isolated from bone marrow metastasis. We previously demonstrated that these subclones possess all the components for an autocrine action of VIP [16]. The SH-SY5Y subclone exhibits a neuroblastic phenotype [17] and VIP treatment for 48 h was recently shown to induce a stable neurite-like outgrowth process in these cells [18]. In the present study, we examined the morphological effects of a short-term VIP treatment on SH-SY5Y cells and investigated the signaling pathways involved.

The mitogen-activated protein kinase (MAPK) or ERK pathway is frequently activated in differentiating cells. However, it is now well known that this signaling pathway is not the only one to be able to induce neurite outgrowth and neuronal differentiation. Thus in the rat pheochromocytoma cell line PC12, another well-characterized cell model used to study neuronal differentiation pathways, both VIP and PACAP induce neurite outgrowth [13,19–21]. However, only PACAP-induced neurite outgrowth has been linked to ERK activation [20,21]. VIP also induces ERK1/2 activation in PC12 cells but no correlation has been reported between ERK activation and the differentiation processes induced by this neuropeptide [13].

It has been demonstrated that an increase in intracellular cAMP content could activate the MAPK/ERK cascade in particular cell types [19,22–27] and that SH-SY5Y cells respond to cAMP by developing neurite-like extensions [28]. In addition, we previously reported that VIP triggered an increase in cAMP intracellular content in these cells [11]. In the present study, we therefore investigated whether VIP could induce neurite morphological changes in SH-SY5Y cells and, if so, whether ERK activation was required for the process. Changes in the actin cytoskeleton are essential determinants of neurite genesis and remodeling, and Rho family GTPases have been assigned important roles in these processes [29,30]. Therefore, we also investigated a possible regulation of Rho GTPase activities by VIP.

We found that VIP induces neurite remodeling characterized by development of varicosities along the neurites and extension of the neurite network. Data suggest a key role for Cdc42 GTPase in neurite remodeling promoted by VIP in SH-SY5Y neuroblastoma cells, while the Ras/ERK pathway is not required for this process.

## Materials and methods

### Materials

### Reagents

Aprotinin, bestatin, antipain, pepstatin, leupeptin, *N*-octylglucopyranoside, actinomycin D, and sodium orthova-

nadate were purchased from Sigma. The MEK inhibitor U0126 was from Calbiochem. ECL+ detection system and Glutathione Sepharose® were from Amersham Biosciences. Immobilon-P membranes were from Millipore Corp.

### Antibodies

The polyclonal anti-Cdc42 and anti-ERK1 antibodies and the monoclonal anti-phosphorylated forms of ERKs, anti-RhoA, and anti-cK-Ras antibodies were from Santa Cruz. The monoclonal anti- $\beta$ -tubulin antibody was from Sigma. The monoclonal anti-Rac1 antibody was from Transduction Laboratories and the monoclonal anti-HA1 antibody was from Bapco. The anti-mouse and anti-rabbit peroxidase-conjugated antibodies were purchased from Calbiochem. Goat anti-mouse antibody conjugated with Cyanine dye Cy3 was from Caltag.

### Plasmids

The plasmid encoding the GST-B-Raf-RBD protein was described previously [26]. This construct encodes the Ras-binding domain (amino acids 146–227) of quail B-Raf [31] fused to the GST. GST-PAK-CD containing the Rac and Cdc42 binding domain from Human PAK1B was produced as described by Sander et al. [32]. GST-C21 fusion protein containing the Rho binding region from the Rho effector protein Rhotekin was produced according to Sander et al. [33]. The plasmids encoding the Cdc42 wt, Cdc42 N17, Cdc42 L61, Rac1 wt, Rac1 N17, and Rac1 L61-GFP fusion proteins were obtained by subcloning each mutant into the pEGFP C1 vector (Clontech) and were a kind gift from Dr Philippe FORT (CNRS FRE 2593, CRBM, Montpellier). The HA-Ras V12 and HA-Ras N17 plasmids were described previously [26].

### Cell culture, specific cell treatments, and transfection

SH-SY5Y neuroblastoma cells and NIH3T3 fibroblasts were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS) and 100 UI/ml penicillin and 100  $\mu$ g/ml streptomycin (Life Technology) in a humidified atmosphere containing 5% CO<sub>2</sub> in air at 37°C.

In all experiments and before treatment, SH-SY5Y cells were grown near confluence and then depleted of growth factors for at least 72 h. NIH3T3 cells were depleted of growth factors for at least 3 h. Cells were treated with VIP (at indicated concentration), 1  $\mu$ M bradykinin, 0.2 UI/ml insulin, or 10% serum for the indicated times. Cells were incubated with 1  $\mu$ M of the MAPK/ERK kinase (MEK) inhibitor U0126 solubilized in DMSO for at least 30 min before addition of neuropeptide. DMSO was used as control.

To study the effects of Cdc42 and Rac1 mutants on cell morphology, SH-SY5Y cells were seeded in 30-mm glass dishes. After 24 h, cells were transfected in Optimem I medium (Life Technology) with 3  $\mu$ g of DNA and 15  $\mu$ l Lipofectin (Invitrogen) for 8 h. Cells were then starved for

24 h, treated or not with 10 nM VIP for 4 h, and then observed by confocal microscopy. To study the effect of constitutively active Ras (HA-Ras V12) or dominant-negative Ras (HA-Ras N17) expression on neurite remodeling, cells were seeded at density of  $2.2 \times 10^6$  cells on glass coverslips coated with Matrigel basement membrane matrix (BD Biosciences). Cells were transfected and stimulated with VIP as described above and coverslips were then treated for observation of HA-positive cells by immunofluorescence.

#### *Analysis of cell morphology*

To study the effect of VIP on cell morphology, SH-SY5Y cells were seeded on glass coverslips and grown for 24 h in complete culture medium. They were then depleted of FCS for 24 h before treatment with 10 nM VIP and with or without 1  $\mu$ M U0126 pretreatment. Cells were fixed with 4% *para*-formaldehyde in PBS for 20 min at 4 °C and observed by phase-contrast microscopy using a Zeiss Axioplan microscope.

#### *Indirect immunofluorescence*

For immunostaining of HA1-tagged Ras-transfected cells, cells cultured on coverslips were fixed with 4% *para*-formaldehyde in TBS for 5 min at room temperature, washed three times in TBS, and then permeabilized for 3 min in TBS containing 0.05% Triton X-100. Cells were washed three times in TBS, blocked in TBS containing 2% BSA for 30 min at room temperature, and exposed to monoclonal anti-HA1 antibody diluted 1/500 in TBS containing 1% BSA for 2 h at room temperature. A goat anti-mouse conjugated with Cyanine dye Cy3 was used as secondary antibody.

#### *Confocal microscopy and data analysis*

EGFP-labeled cells and HA1-Ras-transfected cells were analyzed by laser scanning confocal microscopy (LSCM) using a BioRad MRC 1024 ES (BioRad, Hemel Hempstead, UK). The confocal unit was connected to an inverted microscope (Olympus IX70, Tokyo, Japan). Fluorescence signal collection, image construction, and scaling were performed through the control software (Lasersnap 3.2; BioRad).

#### *ERK activation assay*

Cells were grown in 25-cm<sup>2</sup> flasks, serum starved for 72 h, and treated with agents as indicated. Following treatments, cells were lysed in electrophoresis sample buffer containing 0.06 M Tris (pH 6.8), 2% SDS, 4.5 M urea, and 5%  $\beta$ -Mercaptoethanol.

Proteins (100  $\mu$ g) were separated on 12% SDS-polyacrylamide gels and transferred to nylon membranes

(Immobilon-P). ERK activation was analyzed by immunoblotting using an activation-specific antibody recognizing the dual phosphorylated forms of both p42 ERK2 and p44 ERK1. Total protein levels were assessed by Western blotting using either a monoclonal anti- $\beta$ -tubulin antibody or a polyclonal anti-ERK antibody that recognizes both ERK1 and ERK2.

#### *Pull-down assay for detection of activated Ras*

Cells were grown in 100-mm dishes, serum starved for 72 h, and treated with agents as indicated. Cells were then washed with ice-cold PBS and lysed for 20 min on ice in buffer containing 50 mM Tris pH 7.5, 15 mM NaCl, 20 mM MgCl<sub>2</sub>, 5 mM EGTA, 1% Triton X-100, 1% *N*-octylglucopyranoside, 1  $\mu$ g/ml bestatin, 1  $\mu$ g/ml antipain, 1  $\mu$ g/ml pepstatin, 5  $\mu$ g/ml leupeptin, 1 mM 4-(2-aminoethyl)-benzene-sulfonyl fluoride (AEBSF). Lysates were clarified by centrifugation at 13,000 rpm for 20 min at 4 °C. Protein extracts (700  $\mu$ g) were incubated with 30  $\mu$ l GST-B-Raf-RBD fusion protein precoupled to glutathione-Sepharose beads for 2 h at 4 °C. GST-B-Raf-RBD fusion protein used to trap active Ras (GTP-bound form) was produced and immobilized on glutathione-Sepharose beads as described previously [26]. After incubation, beads were washed three times in lysis buffer and resuspended in Laemmli sample buffer. Samples were analyzed by electrophoresis on SDS-polyacrylamide gel (15%) followed by transfer to nylon membranes (Immobilon-P). Affinity-purified activated Ras was detected by immunoblotting using a monoclonal anti-K-Ras antibody. Total Ras level was evaluated by Western blotting of whole cell extracts (100- $\mu$ g proteins) using the same antibody.

#### *Pull-down assays for detection of activated Rac1, Cdc42, and RhoA*

Following treatments, cells were lysed for 20 min on ice in buffer containing 50 mM Tris pH 7.4, 150 mM NaCl, 5 mM MgCl<sub>2</sub>, 0.05% NP-40, 0.25% sodium deoxycholate, 0.25% Triton X-100, 0.025% SDS, 10  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml leupeptin, 1  $\mu$ g/ml bestatin, 1  $\mu$ g/ml antipain, 1  $\mu$ g/ml pepstatin, 1 mM AEBSF. Lysates were clarified by centrifugation at 13,000 rpm for 5 min at 4 °C. Proteins (700  $\mu$ g) were incubated with 30  $\mu$ l GST fusion protein precoupled to glutathione-Sepharose beads overnight at 4 °C. GST-PAK-CD fusion protein was used to trap Rac1 and Cdc42 in their GTP-bound active form whereas GST-C21 fusion protein was used to trap active RhoA. After incubation, beads were washed three times in buffer containing 50 mM Tris pH 7.4, 150 mM NaCl, 5 mM MgCl<sub>2</sub>, and 0.05% NP-40, and resuspended in Laemmli sample buffer. Samples were analyzed by electrophoresis on SDS-polyacrylamide gel (15%) followed by transfer to nylon membranes. Affinity-purified activated Rac1, Cdc42, and RhoA were detected by immunoblotting using mono-



clonal anti-Rac1, polyclonal anti-Cdc42, and monoclonal anti-RhoA antibodies. Total Rac1, Cdc42, and RhoA levels were evaluated by Western blotting of whole cell extracts (50- or 100- $\mu$ g proteins) using the same antibodies.

## Results

### *VIP induces early neurite morphological changes in SH-SY5Y neuroblastoma cells*

To investigate the effect of VIP, serum-starved SH-SY5Y cells were treated with 10 nM VIP for 4 h and morphological changes were observed by phase-contrast microscopy. Control cells already presented extended slender and straight neurites with few branches (Fig. 1). After 4 h of treatment, VIP induced an extension of the neuritic network between adjacent cells and branching appeared more pronounced (Fig. 1). In addition, numerous prominent varicosities formed

along the neurites. The varicosities were varying in size with largest ones evoking neurite swelling. These effects were rapid, occurring in less than 3 h of treatment and remained visible after 6 h. However, they were transient and reversible because no difference remained observable between control and VIP-stimulated cells after 24 h of VIP treatment.

### *VIP activates the small G protein Ras*

To determine whether a Ras-mediated signaling pathway may be triggered by VIP treatment, we investigated the potential activation of this small GTPase by the neuropeptide. To this aim, a Ras pull-down assay was performed using the Ras binding domain of B-Raf fused to the GST protein (GST-B-Raf-RBD) to trap Ras in its GTP-bound active form. The pool of active Ras was then detected by Western blotting using an anti-K-Ras antibody (Fig. 2). The amount of active Ras increased as early as 5 min after the onset of the 10-nM VIP treatment. Activation was sustained because it remained detectable after 60 min of neuropeptide treatment.

### *VIP induces a time- and concentration-dependent ERKs activation*

We next examined the ability of VIP to induce ERK activation in SH-SY5Y cells. Starved cells were treated with 10 nM VIP for 5 min and up to 24 h (Fig. 3A). The activation of MAP kinases was estimated using a specific antibody recognizing the phosphorylated thus activated forms of ERKs (Fig. 3A, upper panel). This kinetic study revealed that the two MAP kinases, ERK1 and ERK2, were simultaneously activated by VIP. Activation was first observed after 15 min of treatment and appeared to be maximal after 60 min. Longer stimulation led to a decrease of the activation intensity, but MAP kinase phosphorylation was still detectable after 24 h. The dose-dependent effect of VIP on ERK activation was assessed using concentrations of neuropeptide ranging from 0.01 to 100 nM for 60 min (Fig. 3B, upper panel). ERK activation was reached using at least 10 nM VIP, lower concentrations tested being inefficient.

To analyze the requirement for MEK in ERK activation, a specific MEK inhibitor was used in the VIP-induced ERK activation assay. Cells were pretreated for 30 min with 1  $\mu$ M U0126 (Fig. 3C) before addition or in absence of 10 nM VIP for 60 min. VIP induced an increase in ERK activation as compared to control cells (Fig. 3C, upper panel) and U0126 completely inhibited VIP-induced MAP kinases activation.

### *Neurite remodeling process induced by VIP does not require the Ras/ERK pathway*

Because both Ras and ERK were activated by VIP, we looked for the requirement of this pathway in the morphological changes induced by the neuropeptide. To study the role of Ras on neurite remodeling, SH-SY5Y cells were transfected with EGFP alone or with either constitutively

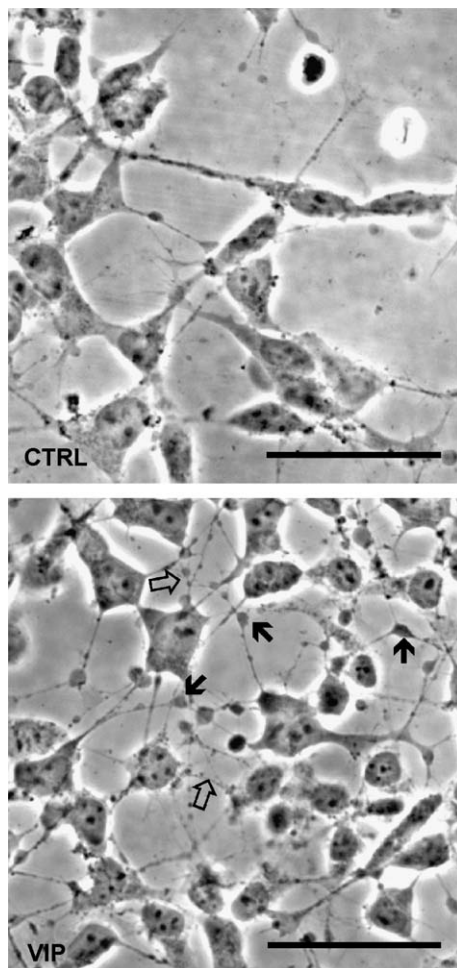


Fig. 1. VIP induces neurite morphological changes in SH-SY5Y neuroblastoma cells. Serum-starved SH-SY5Y cells were unstimulated (CTRL) or treated with 10 nM VIP for 4 h (VIP). The developing morphology was observed by phase contrast microscopy. Black and open arrows indicate varicosities and neurite network, respectively. Scale bars represent 50  $\mu$ m. Micrographs are representative of four independent experiments.

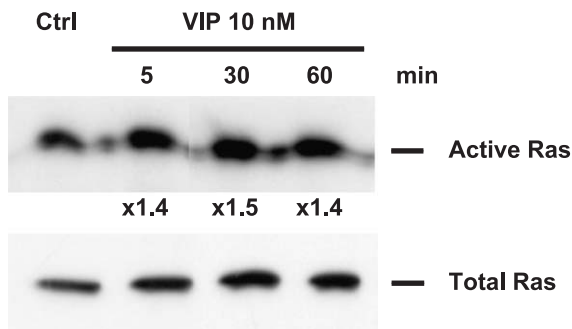


Fig. 2. VIP activates the small G protein Ras. SH-SY5Y cells were treated with 10 nM VIP for the indicated times. Ctrl: unstimulated cells. Pull-down experiments were performed on cell lysates using the GST-B-Raf RBD fusion protein. Activated Ras (Ras-GTP) was detected by Western blotting using anti K-Ras antibody (upper panel). Ras activation was quantified by comparison with the control. Total Ras content was estimated by blotting equal quantity of cell lysates with the same antibody (lower panel). Data shown are representative of three independent experiments.

active Ras V12 or dominant-negative Ras N17 fused to the HA1 epitope. Cells were then treated or not with 10 nM VIP for 4 h. Immunofluorescence was performed using an anti-

HA1 antibody and HA1-Ras-transfected cells were analyzed by LSM (Fig. 4A). Control cells transfected with EGFP extended straight unbranched neurites (panel a). VIP-treated cells developed a winding and branched neuritic tree with short lateral ramifications and several varicosities along the neurites (panel b). No effect on morphology was observed in Ras N17-transfected cells (panel c) when compared to cells transfected with EGFP. In addition, expression of the dominant-negative mutant of Ras did not impair VIP-induced neurite remodeling (panel d), indicating that Ras was not involved in this process. Transfection of constitutively active Ras (Ras V12) significantly altered cell morphology (panel e), inducing neurite elongation and increasing growth cone number. VIP stimulation of Ras V12-transfected cells amplified the neuritic tree complexity especially in growth cones that extended a profusion of growth processes displaying numerous varicosities (panel f). Higher magnification revealed that VIP also induced short and thin lateral outgrowth processes along the neurites as well as numerous filopodial-like protrusions emerging from the growth cones (panel g) and around the cell body (panel h). To investigate

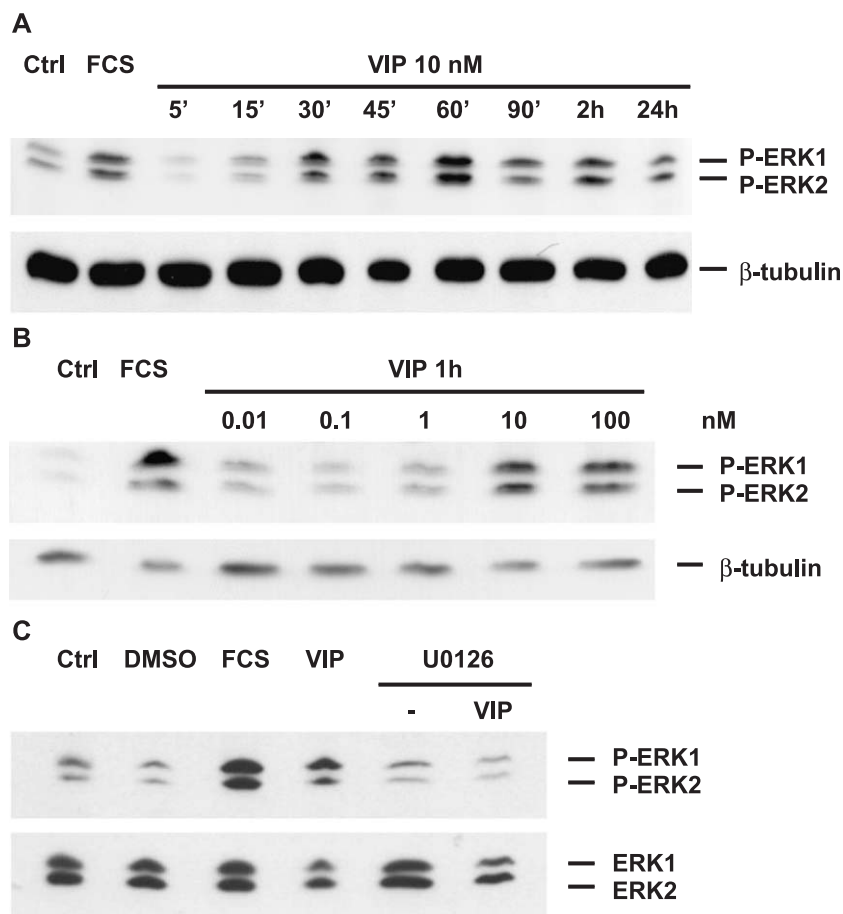
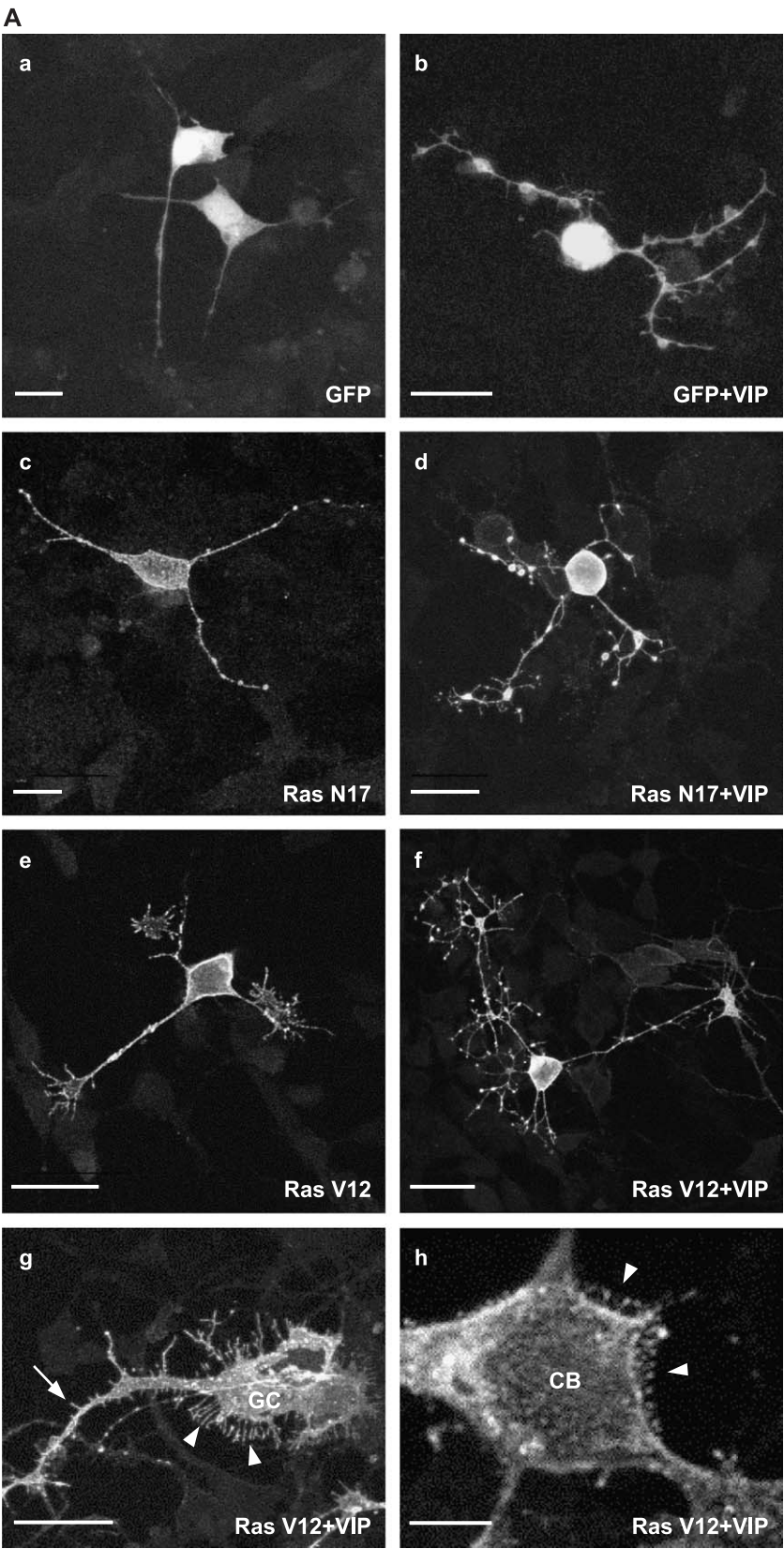


Fig. 3. VIP induces a time- and concentration-dependent ERK activation that requires MEK activity. SH-SY5Y cells were stimulated for various times with 10 nM VIP (A) or with different concentrations of neuropeptide for 60 min (B). Fetal calf serum (10% FCS for 5 min) was used as positive control of ERK activation. Cells were also pretreated with 1  $\mu$ M of the MEK inhibitor U0126 for 30 min before addition of 10 nM VIP for 1 h or in the absence of neuropeptide (C). Ctrl: unstimulated cells. Cells were then lysed in electrophoresis sample buffer. ERK phosphorylation/activation was assessed by Western blotting using an anti phospho-ERKs antibody (A, B, and C: upper panels). Anti- $\beta$ -tubulin antibody (A and B: lower panels) or anti-ERK antibody (C: lower panel) was used on the same blot to verify protein amounts in each sample. Data presented are representative of three independent experiments.

the role of ERK, serum-free cell cultures were pretreated with 1  $\mu$ M of the MEK inhibitor U0126 for 30 min before addition of 10 nM VIP or in absence of the neuropeptide (Fig. 4B). The

MEK inhibitor effect was examined after 4 h of VIP treatment. U0126 alone had no effect on cell morphology because treated cells presented extended long neurites with





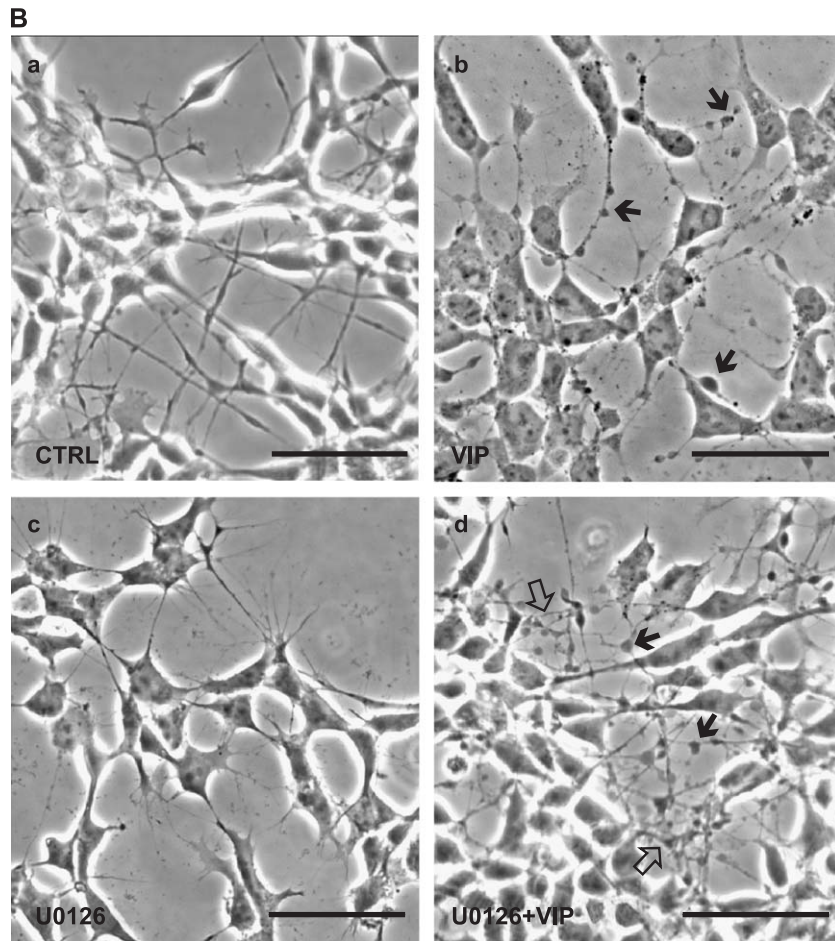


Fig. 4. (A) Ras is not required for the neurite remodeling process induced by VIP. SH-SY5Y cells were transfected with GFP, HA-Ras N17, or HA-Ras V12. After serum starvation for 24 h, cells were unstimulated (a, c, e) or treated with 10 nM VIP for 4 h (b, d, f, g, h). HA-Ras-transfected cells were then treated for indirect immunofluorescence observation. Fluorescent cells were observed by laser scanning confocal microscopy. Lower panels (g and h) focus on lateral neurite outgrowth processes (arrows) and on filopodial-like protrusions (arrowheads) induced by VIP in a growth cone (GC) or on the cell body (CB) of Ras V12-transfected cells. Scale bars represent 20  $\mu$ m (a, b, c, d, e, f) or 5  $\mu$ m (g, h). Micrographs shown are representative of three different experiments. (B) Neurite remodeling process induced by VIP does not require MAP kinase activity. Serum-free cultures of SH-SY5Y cells were treated with 10 nM VIP (b) or pretreated with 1  $\mu$ M of the MEK inhibitor U0126 for 30 min before 10 nM VIP treatment (d). a: unstimulated cells. c: cells only treated with 1  $\mu$ M U0126 inhibitor. The developing morphology was observed by phase contrast microscopy after 4 h VIP treatment. Black arrows indicate varicosities and open arrows indicate the neurite network. Scale bars represent 50  $\mu$ m. These micrographs are representative of three different experiments.

only few branches like control cells (panels c and a, respectively). Pretreatment of cells with the MEK inhibitor did not prevent the VIP-induced neurite modifications (panel d). The same morphological changes, including an increase in neuritic branching and development of varicosities along the neurites, were observed in VIP-treated cells either in the absence or presence of U0126. No modification of VIP effects could be observed using higher concentrations of inhibitor (data not shown).

#### *VIP induces differential effects on Rho family GTPases*

Small G proteins of the Rho family, RhoA, Rac1, and Cdc42 are involved in regulation of actin cytoskeleton and are specially implicated in neuroblastoma cell differentiation [34,35]. To check the potential implication of Rho GTPases in VIP-induced signaling pathways, pull-down assays were

performed. SH-SY5Y cells were treated with 10 nM VIP for 5 min and up to 30 min in the absence of serum (Fig. 5). Known activators of each GTPase were used as positive controls in the pull-down assays, such as 1  $\mu$ M bradykinin (Bdk) for Cdc42, 0.2 UI/ml insulin (Ins) for Rac1, and 10% FCS for RhoA. Activation was estimated using the fusion proteins GST-PAK-CD for Cdc42 and Rac1 and GST-C21 for RhoA as baits to trap the Rho GTPases in their active forms. Rho family proteins were then analyzed by Western blotting using appropriate antibodies. VIP treatment induced an increase in active GTP-bound Cdc42 amount (Fig. 5A, upper panel). Strong activation occurred after 5 min of treatment ( $\times 3.5$ ) and remained visible after 30 min of stimulation. In contrast, a decrease in active Rac1 amount was observed upon VIP treatment (Fig. 5B, upper panel), reaching 50% of the basal level after 15 min. Insulin was only able to induce a weak Rac1 activation in SH-SY5Y cells. The RhoA-GTP amount



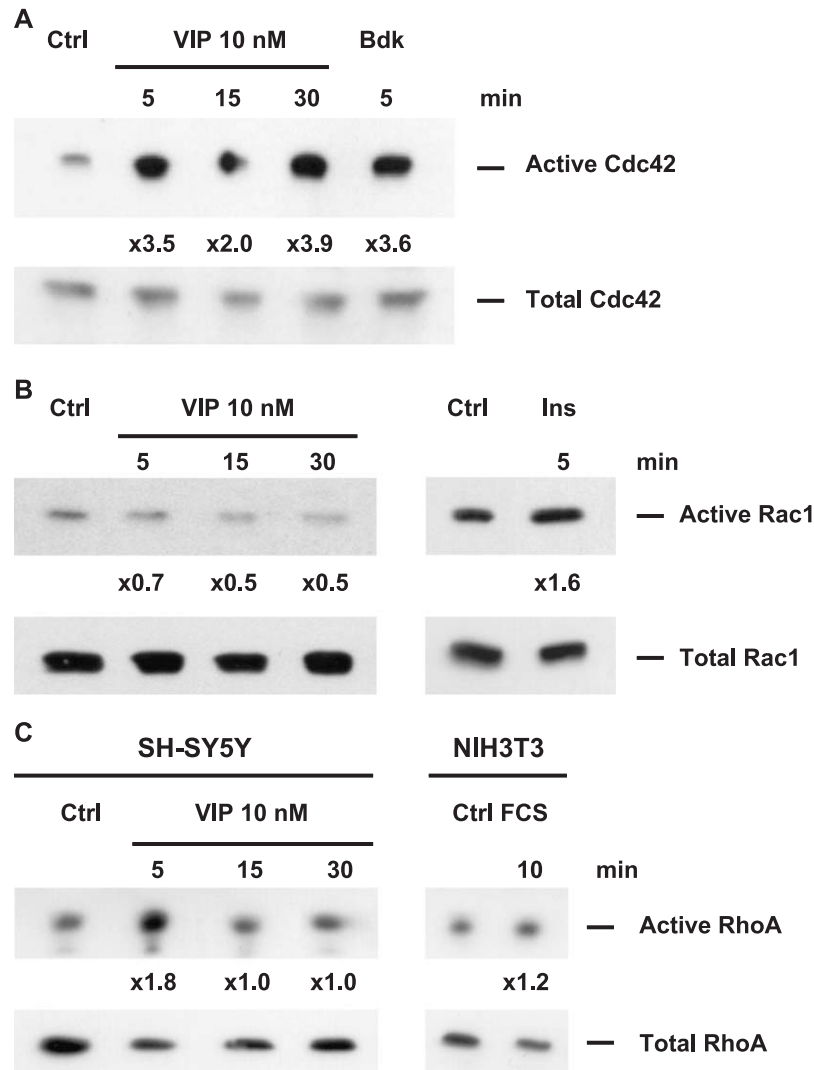


Fig. 5. VIP induces differential effects on Rho family GTPases. SH-SY5Y cells were treated with 10 nM VIP for the indicated times, 1  $\mu$ M bradykinin (Bdk) for 5 min, 0.2 UI/ml insulin (Ins) for 5 min. NIH3T3 cells used as control were treated with 10% fetal calf serum (FCS) for 10 min. Ctrl: unstimulated cells. Cell lysates were subjected to pull-down experiments using the GST-PAK-CD fusion protein (A and B) or GST-C21 fusion protein (C). Activated Cdc42 (Cdc42-GTP), Rac1 (Rac1-GTP), and RhoA (RhoA-GTP) were detected by Western blotting using specific Cdc42, Rac1, or RhoA antibodies, respectively (A, B, and C: upper panels). Activation or inhibition was quantified by comparison with the control. Total Cdc42, Rac1, and RhoA contents were estimated by blotting equal quantity of cell lysates with the same antibodies (A, B, and C: lower panels). Data presented are representative of three independent experiments.

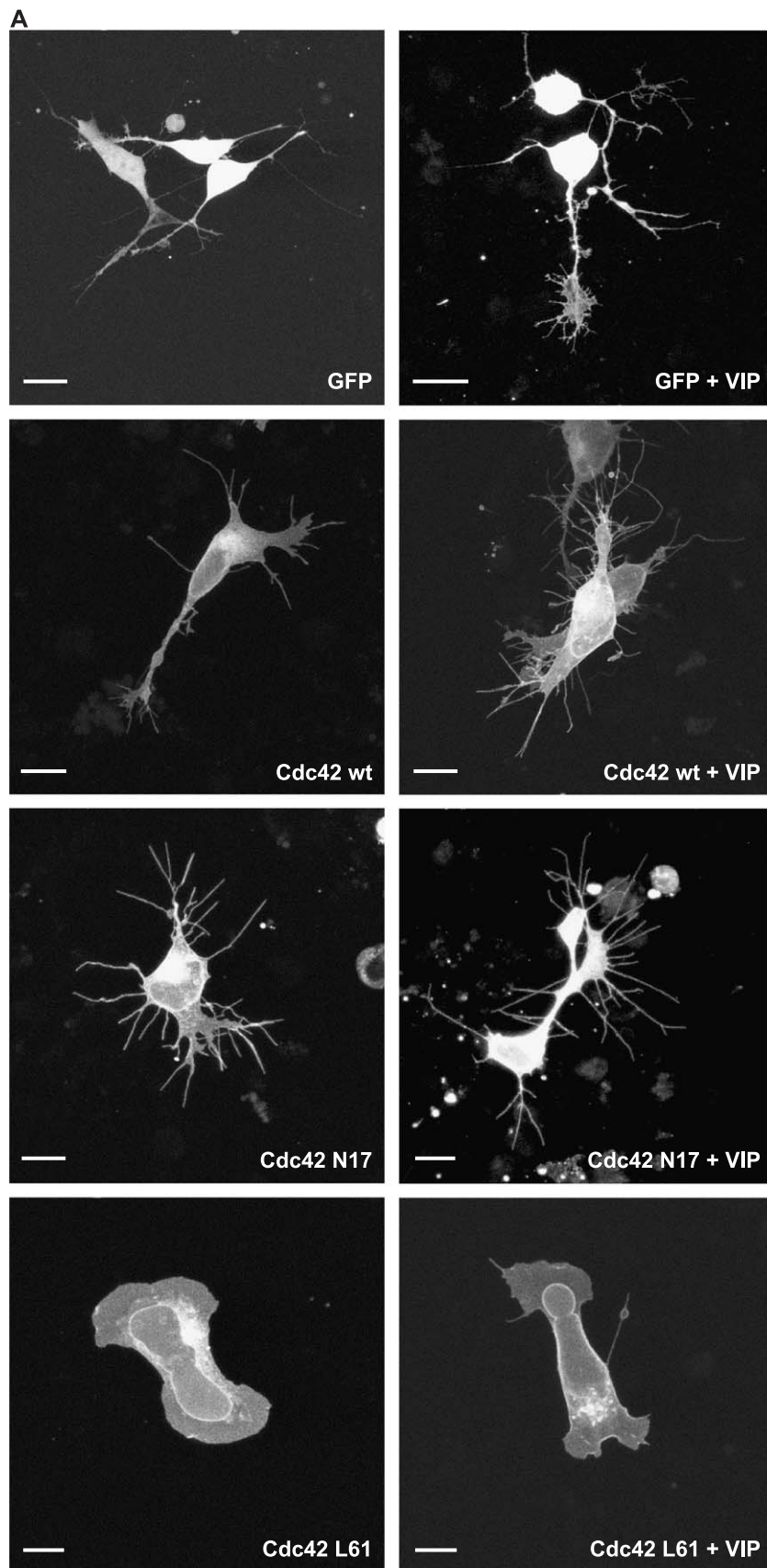
first increased after 5 min of treatment and then returned to its basal level after 15 min of VIP treatment (Fig. 5C, upper panel). NIH3T3 cells were used as positive control of RhoA activation by FCS because serum was not able to induce activation of this GTPase in SH-SY5Y cells.

#### *Dominant-negative or constitutively active mutants of Cdc42 and Rac1 block the neurite morphological modifications induced by VIP*

To investigate the potential role of Cdc42 and Rac1 GTPases in the morphological processes induced by VIP,

SH-SY5Y cells were transfected with positive and negative mutants of these GTPases fused to EGFP. Cells were then treated or not with 10 nM VIP for 4 h and analyzed by LSCM (Figs. 6A and B). Two major phenotypes could be distinguished: bipolar cells with axon-like process and growth cone or non-polarized cells characterized by neurites all around their cell body. Control cells transfected with pEGFP exhibited some long and slender neurites with few branches. VIP treatment (GFP + VIP) induced an increase in the complexity of the neuritic network, mostly characterized by short lateral ramifications growing, an increase of the occurrence of growth cones (see Fig. 6A), and the develop-

Fig. 6. Dominant-negative or constitutively active mutants of Cdc42 and Rac1 block the neurite morphological modifications induced by VIP in SH-SY5Y cells. SH-SY5Y cells were transfected with GFP, GFP-Cdc42 wt, GFP-Cdc42 N17, or GFP-Cdc42 L61 (A), GFP, GFP-Rac1 wt, GFP-Rac1 N17, or GFP-Rac1 L61 (B). After serum starvation for 24 h, cells were treated with 10 nM VIP for 4 h and then observed by laser scanning confocal microscopy. Micrographs shown are representative of three independent experiments and illustrate the predominant phenotypes observed in cell cultures. Scale bars represent 20  $\mu$ m.



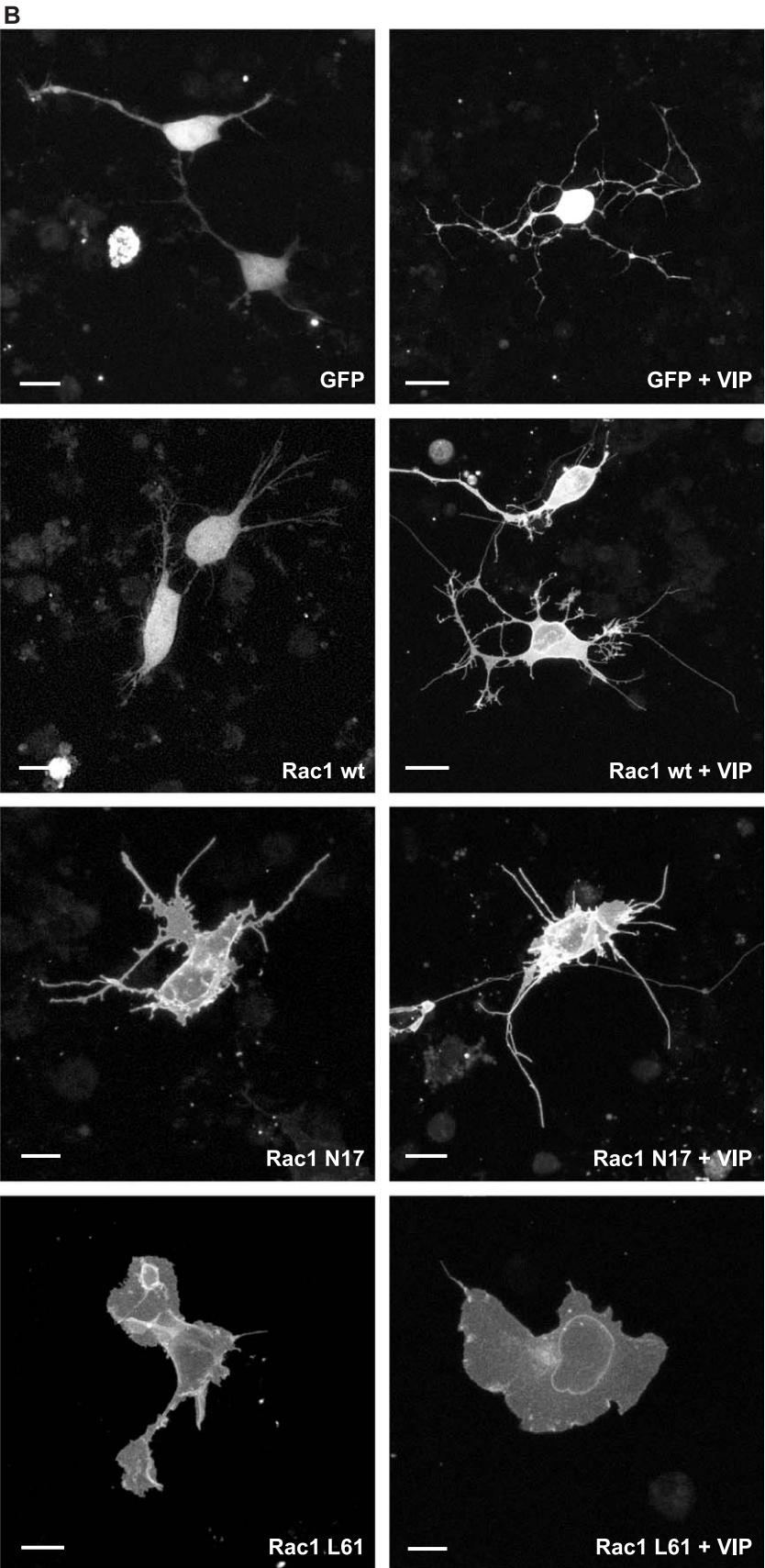


Fig. 6 (continued).

ment of dendritic tree-like structures (see Fig. 6B). Depending on the original phenotype, VIP-induced remodeling was varying: bipolar cells were engaged in growth cone formation and short lateral branching, while nonpolarized cells developed a widespread arborization with numerous varicosities. GFP-Cdc42 and GFP-Rac1 wild-type-expressing cells (Cdc42 wt and Rac1 wt) displayed a morphology quite similar to that of pEGFP-transfected cells. Following VIP stimulation, these transfected cells (Cdc42 wt + VIP and Rac1 wt + VIP) exhibited enhanced branching as mentioned above. New phenotypes were observed when SH-SY5Y cells were transfected with dominant-negative or constitutively active mutants of Cdc42 and Rac1. Both GFP-Cdc42 N17- and GFP-Rac1 N17-expressing cells showed similar phenotypes characterized by the presence of several long and threadlike processes with few branching all around the cell body. Interestingly, no additional morphological effect was observed after VIP stimulation (Cdc42 N17 + VIP, Rac1 N17 + VIP). In contrast, GFP-Cdc42 L61- and GFP-Rac1 L61-transfected cells developed large lamellipodia. It should be noticed that only few cells harbored lamellipodia and neurites simultaneously. VIP treatment (Cdc42 L61 + VIP, Rac1 L61 + VIP) did not counteract lamellipodia formation and classical VIP-induced remodeling was not observed.

## Discussion

The functions of the ubiquitous neuropeptide VIP as a regulator of proliferation or differentiation in normal and tumoral cells are well known. However, the intracellular signaling pathways induced by this neuropeptide are not yet clearly elucidated [5–11,36,37]. In the present work, we used the human neuroblastoma cell line SH-SY5Y, a well-characterized model of neuronal differentiation, to examine the early morphological effects of VIP and to investigate the signaling pathways triggered by neuropeptide stimulation. In this way we expected to gain new information about the fine regulation of neuronal plasticity by VIP neuropeptide.

Within 4 h, a remodeling of SH-SY5Y cell projections was induced by 10 nM VIP, namely an extension of the neurite network between adjacent cells and development of varicosities varying in size along the neurites. EGFP expression coupled to laser scanning confocal microscopy analysis allowed a fine resolution of these modifications, especially the formation of new neurite branching, which markedly increased the complexity of the dendritic tree. These early induced processes were transient because they were no longer observed after 24 h of neuropeptide stimulation. Indeed, this remodeling should not be considered as a differentiation process per se but only as morphological changes underlying neuronal plasticity. In this way, it differed from the stable neuritogenesis produced by VIP in these cells, which needed both serum starvation for 6–7 days and a 48-h treatment with the neuropeptide

[18]. A similar neurite remodeling could be produced in the presence of 10  $\mu$ M forskolin, an activator of adenylate cyclase (data not shown). Since we previously reported that VIP triggered an increase in cAMP intracellular content in SH-SY5Y cells [11], it is postulated that the VIP-induced morphological changes may be mediated by a cAMP signaling pathway. Interestingly, it should be noted that a cAMP-activated pathway regulating neuronal differentiation has been recently identified in SH-SY5Y cells [28].

In the pheochromocytoma cell line PC12, which derives from neural crest-like SH-SY5Y cells, both VIP and PACAP have been previously shown to induce neuritogenesis [13,19–21] and to activate MAP kinases [20,21,38]. Therefore, we first investigated whether Ras and ERKs could be potential targets of VIP's effect in SH-SY5Y neuroblastoma cells. Using pull-down experiments, we found that Ras was rapidly but weakly activated. Concomitantly, VIP also triggered sustained ERK activation. We then investigated whether Ras or MAPK could be required for the VIP-mediated neurite remodeling process. We found that expression of Ras N17-dominant negative mutant had no effect on neurite morphology in SH-SY5Y cells and did not prevent the VIP effect, strongly suggesting that Ras activity was not required for neurite remodeling. In contrast, the constitutively active Ras V12 mutant induced specific changes, namely neurite elongation and an increase in the growth cone number. In addition, the overall neuritic-tree complexity was even more pronounced when Ras V12-transfected cells were stimulated with VIP. Therefore, these findings further confirm that the VIP-mediated neurite remodeling is likely not dependent on Ras activity.

Similarly, the MEK inhibitor U0126 completely inhibited the VIP-induced ERK activation but did not modify the morphological changes induced by the neuropeptide. Indeed, extension of the neurite network and formation of varicosities along the neurites induced by VIP treatment were observed both in the absence and presence of the inhibitor. This result suggests that VIP-induced neurite remodeling does not require ERK activity. Interestingly, Olsson and Nanberg [39] have investigated the role of ERK and Ras during neuronal differentiation of SH-SY5Y cells after phorbol ester treatment. As observed in the present study, they reported a sustained activation of ERK and pointed out that expression of constitutively active Ras V12 induced a pronounced formation of neurites with growth cones. However, they demonstrated that ERK activation was only involved in the gene induction process but was not required for neurite outgrowth and that the mechanism by which phorbol ester stimulated neurite formation was distinct from that of Ras-induced outgrowth. In this way, our results represent another example of neurite morphological remodeling that proved to be independent of both Ras and ERK activities.

Reorganization of both the actin cytoskeleton and microtubules is an essential phenomenon accompanying neuronal changes such as neurite development or collapse.



Rho family GTPases including RhoA, Rac1, and Cdc42 play key roles in the regulation of these processes [29,30,34,40]. In neuroblastoma and PC12 cell lines, Cdc42 and Rac1 control neurite extension [41–45], and conversely, RhoA competes with Cdc42 and Rac1 in this process [42,43,46]. Cdc42 and Rac1 also promoted formation of filopodia and lamellipodia influencing growth cone morphology and guidance [42]. Induction of neurite remodeling by VIP in SH-SY5Y cells led us to look for a potential implication of Rho family GTPases. Using pull-down assays, we demonstrate that VIP induced a rapid and strong activation of Cdc42. In contrast, the neuropeptide appeared to inhibit the small GTPase Rac1. Considering the major Cdc42 activation observed, we hypothesized that this GTPase could be responsible for the remodeling triggered by VIP stimulation and therefore we used constitutively active or dominant-negative mutants of Cdc42 and Rac1 to test this hypothesis. Overexpression of wild-type GTPases did not significantly modify the phenotype observed in GFP-transfected cells both in the absence and presence of VIP stimulation. Considering the increase in the expression level of GTPases in transfected cells, these observations would suggest the existence of a strong intrinsic regulation of Cdc42 and Rac1 activities in SH-SY5Y neuroblastoma cells. In contrast, overexpression of dominant-negative or constitutively active Cdc42 or Rac1 proteins resulted in two peculiar phenotypes. Dominant-negative Cdc42/Rac1-transfected cells displayed long and threadlike neurites all around the cell body, whereas constitutively active Cdc42/Rac1-transfected cells acquired lamellipodia structures. Surprisingly, overexpression of constitutively active Cdc42 induced a lamellipodial phenotype, evoking a typical Rac-dependent effect and suggesting a sequential activation of this GTPase downstream of Cdc42. This finding was not in accordance with VIP effect because VIP stimulation of SH-SY5Y cells resulted in Cdc42 activation only, but rather seemed to inhibit Rac1. According to the well-known signaling hierarchies that exist between the activation state of the Rho family GTPases [47], our findings strongly suggested that the lamellipodium formation observed in constitutively active Cdc42-transfected cells would result from Cdc42-triggered activation of Rac1.

A major finding was that transfection of SH-SY5Y cells with dominant-negative N17 mutant of Cdc42 completely blocked VIP effects, suggesting that Cdc42 is indeed required for VIP-induced neurite remodeling. Consistently, we observed a substantial activation of Cdc42 in cells stimulated with VIP as well as VIP-induced growth of typical Cdc42-dependent filopodial protrusions, further suggesting a fundamental role for this GTPase.

Although VIP did not induce Rac1 activation, the dominant-negative mutant of Rac1 also seemed to prevent the neuropeptide effect on neurites. One possibility would be that a permissive and reduced Rac1 activity is yet needed to enable the VIP-mediated neurite changes. Such a requirement for low level of active Rac1 has been already

demonstrated for Ras-transformed cells to proliferate [48]. In this hypothesis, further activation of Rac1 by VIP would not be required for neurite remodeling, which is consistent with the Rac1 pull-down assay data. Another hypothesis would be that a turnover of the two GTPases is needed to accomplish the remodeling program of VIP. In fact, constitutive activation or inhibition of Rac1 and Cdc42 induces particular phenotypes that prevent any further VIP effect on cellular morphology.

Taken together, our findings bring new insights in the VIP signaling pathways and the VIP-associated remodeling process in neuroblastoma cells. VIP was able to induce Ras and ERK activation in SH-SY5Y cells, but neither Ras nor ERK was required for VIP-induced morphological changes. In addition, we showed that VIP induces a strong activation of Cdc42. To our knowledge, this is the first report showing a regulatory effect of a neuropeptide of the VIP family on the activity of Rho GTPases and demonstrating that Cdc42 may play a key role in the cytoskeleton reorganization accompanying VIP-induced neurite remodeling.

## Acknowledgments

We would like to thank Dr R. Busca and C. Peyssonnaud for their help with pull-down assays, A. Cantereau for technical assistance with confocal microscopy, and J. Habrioux for desktop publishing. We are grateful to Dr. J. Collard for GST-PAK-CD and GST-C21 plasmids and Dr. P. Fort for pEGFP C1 expression vectors. This work was supported by CNRS and C. Alleaume was the recipient of PhD fellowship from the “Région Poitou-Charentes”.

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