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ORIGINAL ARTICLE

Effects of the Vasoactive Intestinal Peptide (VIP) and Related Peptides on Glioblastoma Cell Growth in Vitro

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Abstract

The growth rate of numerous cancer cell lines is regulated in part by actions of neuropeptides of the vasoactive intestinal peptide (VIP) family, which also includes pituitary adenylate cyclase-activating peptide (PACAP), glucagon, and peptide histidine/isoleucine (PHI). The aim of this work was to investigate the effect of these peptides on the growth of the rat glioblastoma cell line C6 in vitro. We also sought to determine which binding sites were correlated with the effects observed. Proliferation studies performed by means of a CyQuant™ assay showed that VIP and PACAP strongly stimulated C6 cell proliferation at most of the concentrations tested, whereas PHI increased cell proliferation only when associated with VIP. Two growth hormone–releasing factor (GRF) derivatives and the VIP antagonist hybrid peptide neurotensin-VIP were able to inhibit VIP-induced cell growth stimulation, even at very low concentrations. Binding experiments carried out on intact cultured C6 cells, using ^{125}I-labeled VIP and PACAP as tracers, revealed that the effects of the peptides on cell growth were correlated with the expression on C6 cells of polyvalent high-affinity VIP-PACAP binding sites and of a second subtype corresponding to very high-affinity VIP-selective binding species. The latter subtype, which interacted poorly with PACAP with a 10,000-fold lower affinity than VIP, might mediate the antagonist effects of neurotensin-VIP and of both GRF derivatives on VIP-induced cell growth stimulation.

Index Entries: C6 glioblastoma; vasoactive intestinal peptide (VIP); cell growth; cancer; VIP analog; VIP antagonist.

Introduction

Primary central nervous system (CNS) tumors account for approx 2% of all adult malignancies but are responsible for approx 7% of years of life lost owing to malignancy prior to age 70. Within adult CNS tumors, gliomas represent 40–67% of primary tumors. In children, 20% of all malignancies diagnosed before age 15 are primary CNS tumors, making them the second most frequently diagnosed childhood malignancy; and it is estimated that gliomas comprise 67% of such tumors (Bohnen et al., 1997). Of all primary CNS malignancies, glioblastoma, a highly malignant glioma, has the poorest prognosis, with the median survival limited to 7–9 mo (Ammirati et al., 1987). Even with aggressive surgical resection followed by adjuvant therapy, such as radiotherapy, immunotherapy, and chemotherapy, the great majority of patients diagnosed with these high-grade gliomas succumb to their disease. The

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prognosis for these brain tumors has not changed significantly in the last decade, making the search for new treatments particularly pertinent.

Neuropeptides, such as the 28-amino-acid peptide vasoactive intestinal peptide (VIP) (Said and Mutt, 1970), fulfill an important role in the regulation of normal neuronal development. Identified very early in neural development, VIP appears to regulate both proliferation and differentiation of neuronal precursors in vitro and in vivo, and is also involved in growth, differentiation, and maintenance of neurons, among a broad range of biological activities (for reviews, *see* Gozes and Brenneman, 1993; Gressens et al., 1993; Muller et al., 1995). It has also been demonstrated that VIP has neuroprotective properties that influence the survival of activity-dependent neurons in the CNS. These properties are mediated in part through the action of glial-derived substances released by VIP, including the activity-dependent neurotrophic factor (ADNF), a novel neuroprotective protein that shares structural similarities to heat shock protein 60 (hsp 60) (Brenneman and Gozes, 1996; Gozes and Brenneman, 1996, Brenneman et al., 1998), hsp 60 itself (Bassan et al., 1998) or the activity-dependent neurotrophic protein (ADNP), another glial mediator of VIP-associated neuroprotection (Bassan et al., 1999; Offen et al., 2000). Moreover, neuropeptides have been implicated in the progression and differentiation of malignant tumors of the CNS and peripheral nervous system. Although extensive data are available on the expression of these regulatory peptides and their respective receptors in adult nervous system tumors and in neuroblastoma of childhood (Muller et al., 1995; Vertongen et al., 1997; Waschek et al., 1997; Lelièvre et al., 1998b), little is known about their occurrence and significance in other neuronal malignancies.

These observations, together with the neuroendocrine nature of glioma and the identification of VIP in gliomas (Bateman et al., 1986), provide the basis for our hypothesis that VIP might regulate proliferation in glioma via interaction with high-affinity receptors.

In this study, we evaluated the ability of VIP to modulate cell growth of the C6 rat glioblastoma cell line (Benda et al., 1968) in vitro. We compared the effects obtained with this peptide with those of VIP structural analogs, Pituitary adenylate cyclase-activating peptide-27 (PACAP-27), glucagon, and peptide histidine/isoleucine (PHI) amide, which are able to regulate the proliferation rate of numerous cell lines (Pavelic and Pavelic, 1980; Moyer et al., 1985; Vertongen et al., 1996; Lelièvre et al., 1998b). Furthermore, we investigated whether the VIP effects were sensitive to antagonists demonstrated to interact with VIP binding sites, such as the D-amino acid-substituted growth hormone releasing factor [N-Ac-Tyr^1-D-Phe^2]-GRF (1–29)-NH_2 (Waelbroeck et al., 1985), [N-Ac-Tyr^1-D-Arg^2]-GRF (1–29)-NH_2 (Robberecht et al., 1985), and the chimeric peptide neurotensin (6–11)-VIP (7–28) (Gozes et al., 1995), designed to maintain the binding capacity of the parent molecule VIP while loosing the agonistic properties. Finally, we determined the pharmacological properties of the VIP or PACAP binding sites that may mediate the effects observed on cell growth.

Materials and Methods

Chemicals

C6 rat glioblastoma cells (Benda et al., 1968) were purchased from the European Collection of Animal Cell Culture (ECACC) (Salisbury, UK). Glutamax-I Dulbecco's modified Eagle's medium (DMEM) (4.5 g/L glucose, with sodium pyruvate), penicillin-streptomycin (10,000 U/mL penicillin G sodium and 10,000 µg/mL streptomycin sulfate in 0.85% saline), trypsin-EDTA solution (0.5 g/L trypsin and 0.2 g/L EDTA in Puck's medium), and fetal bovine serum (FBS) were obtained from GIBCO BRL (France). Human VIP, PACAP-27, glucagon, PHI, and [N-Ac-Tyr^1, D-Phe^2]-GRF (1–29) amide were from Neosystem (France). [N-Ac-Tyr^1-D-Arg^2]-GRF (1–29)-NH_2 and neurotensin (6–11)-VIP (7–28) were from Sigma Chemical (France).

CyQuant™ Cell proliferation assay kit was purchased from Molecular Probes (USA). ^{125}I-labeled Na (2200 Ci/mmol) was provided by NEN Du Pont (France). Chloramine T, sodium metabisulfite, trifluoroacetic acid (TFA), bovine serum albumin (BSA), bacitracin, and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma Chemical (France).

Peptide Radioiodination

Vasoactive intestinal peptide or PACAP was radioiodinated using the chloramine T method, as described previously (Martin et al., 1986; Pineau et al., 2001). Briefly, the reaction was initiated by the addition of 25 µL of a 3 mg/mL chloramine T solution (prepared in a 0.2 *M* sodium phosphate buffer [pH 7.6]) to 15 µL of a 10^{-4} *M* aqueous solution of peptides mixed with 5 µL of ^{125}I-labeled Na solution

(~0.45 mCi). After 1 min, the reaction was stopped with 25 µL of 2 mg/mL sodium metabisulfite prepared in the same buffer. The radioiodination mix was then passed on a C_{18} Sep-Pak column (Waters Associates) using 10 mL of a 0.1% TFA aqueous solution as eluent to discard free radioiodine. The ^{125}I-labeled peptides were then recovered using 2.5 mL of 85:15 acetonitrile/0.1% TFA in water as eluent. They were further purified by reverse-phase high performance liquid chromatography (HPLC), using a 5-µm Vydac C_{18} column (Interchrom, France). Elution was conducted for 32 min with a 0–85% linear gradient of acetonitrile in H_2O/0.1% TFA. The fractions corresponding to the monoiodinated form of the peptide were dried under nitrogen and stored at –20°C.

Cell Culture

C6 rat glioblastoma cells (used between passages 7 and 25) were grown as monolayer cultures in 75-cm^2 flasks (Falcon) in DMEM supplemented with 3% (v/v) FBS and penicillin-streptomycin, in a humidified atmosphere of 95% air/5% CO_2 at 37°C. Medium was changed twice a week, and cells were subcultured every seventh day. For subcultures, cells were harvested in trypsin-EDTA solution (0.05% and 0.53 m*M*, respectively) in Puck's medium, for 10 min at 37°C. Experiments were conducted with cells in exponential growth phase.

Effect of VIP and Related Peptides on Cell Growth

The effects of VIP and the VIP analogs PACAP-27, PHI, and glucagon were tested in vitro on C6 cells. Furthermore, the effects of three antagonists demonstrated to interact with VIP receptors were also checked on C6 cell growth:

- [N-Ac-Tyr^1, D-Phe^2]-GRF (1–29) amide. This antagonist was shown to selectively inhibit both VIP and GRF-stimulated adenylate cyclase activities in rat pancreatic plasma membranes (Waelbroeck et al., 1985; Robberecht et al., 1986) and is thus referred to here as VIP antagonist to facilitate the reading of this article and for the purpose of this article only.
- [N-Ac-Tyr^1-D-Arg^2]-GRF (1–29)-NH_2. This antagonist was able to inhibit the GRF-stimulated adenylate cyclase in adenopituitary membranes (Robberecht et al., 1986), and is referred to here as GRF antagonist.
- Neurotensin (6–11)-VIP (7–28). Acute administration of this molecule resulted in the blockade of VIP-mediated potentiation of sexual behavior in the adult rodent and in severe microcephaly during gestation once administered to pregnant mice (Gozes et al., 1995).

Cells were seeded in quintuplicate at a density of 7500 cells/well in 96-well culture plates in 200 µL of culture medium. At 24 h after plating, the medium was removed, and fresh serum-free culture medium containing the peptide tested at final concentrations of 10^{-15}–10^{-8} *M* was added. The effect induced by the association of VIP (10^{-13} *M*) and PHI, or VIP (10^{-9} *M*) and each antagonist, was also tested. Control cultures were treated with medium without any peptide addition or with VIP alone (10^{-9} *M*) in the case of the antagonist assay. The cells were then further incubated for 3 d. At the end of the treatment, medium was removed and the cells were frozen until the cell-counting assay. Cell growth was assessed by using the CyQuant cell proliferation assay kit, based on the use of the green fluorescent CyQuant GR dye, which exhibits strong fluorescence enhancement when bound to cellular nucleic acids. Frozen cells were simply thawed and lysed by addition of a buffer containing the CyQuant GR dye (200 µL/well) at room temperature and protection from light. Fluorescence was then measured directly using a fluorescence microplate reader (Fluorocount Packard) with filters appropriate for 480-nm excitation and 520-nm emission maxima. Data were expressed in percentage of gain or loss of cells compared to control, set to 0%.

Binding Studies

The receptor binding affinity of VIP, VIP-related peptides, and antagonists in C6 rat glioblastoma cells was measured in displacement experiments based on competitive inhibition of radiolabeled peptide binding using various concentrations of unlabeled peptide, as previously reported (Muller et al., 1989). Briefly, cells (200,000 cells/well) were seeded in 24-well plates and cultured for 3 d. Culture medium was then removed, and the cells were incubated for 2 h at 13°C, in the presence of 50 p*M* ^{125}I-labeled, VIP or PACAP and increasing concentrations of VIP, PHI, PACAP-27, or antagonists in DMEM/150 m*M* HEPES, pH 7.4, containing 1% BSA, 0.1% bacitracin, and 150 µ*M* PMSF. Binding reactions were stopped by cooling the dishes on ice. Cells were rinsed three times with 1 mL cold phosphate-buffered saline (PBS)/0.1% BSA and lysed with 500 µL of 0.5 *M* NaOH. Radioactivity of the cell lysates was quantified with a Cobra II Auto-Gamma Counting System (Packard, Downers Grove, IL). Specific binding was calculated as the difference between the mean determination of total binding and binding in the presence of excess native VIP or PACAP-27 (10^{-7} *M*). The

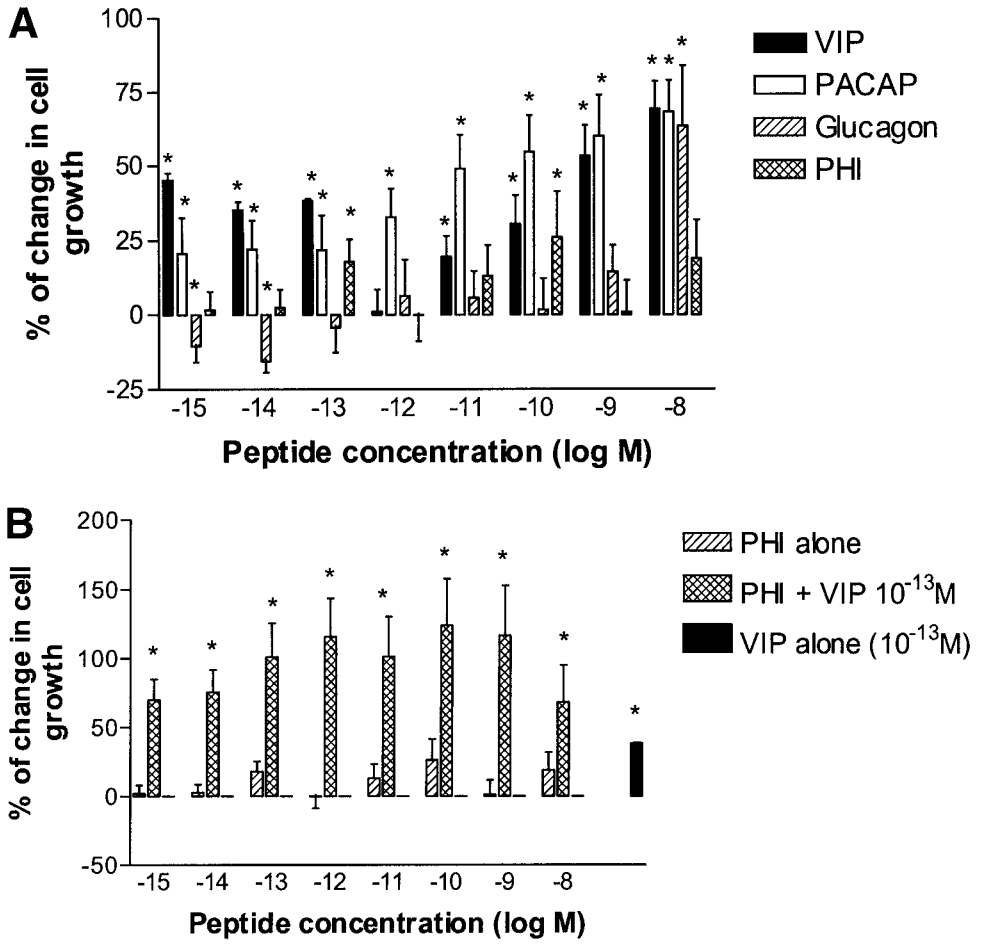

Fig. 1. Effect of increasing concentrations of exogenous VIP and VIP-related peptides (PACAP-27, PHI, and glucagon) on the proliferation of C6 cells in culture, each acting alone **(A)**. **(B)** Combined action of VIP (10^{-13} *M*), together with increasing concentrations of PHI. Cell growth was expressed as the percentage of variation of cell number compared to cell number observed without treatment (control) (0%). Data are the mean ± S.E.M. of three independent experiments, each performed in quintuplicate. (*) $p < 0.05$ vs untreated cells.

fitting of the data by nonlinear regression was computed according to a Hill equation using Graphpad™ Prism software (San Diego, CA) in order to determine two parameters: (1) the concentration of unlabeled peptide giving half-maximal inhibition of the radiotracer binding (IC_{50}); and (2) the total number of sites/cell interacting with a given radiotracer (B_{max}).

Statistical Analysis

Results are expressed as means ±S.E.M., and statistical significance was determined by one-way analysis of variance (ANOVA), followed by Bonferroni's test. Differences were considered significant when $p < 0.05$, very significant when $p < 0.01$, and highly significant when $p < 0.001$. Data were the meaning of three independent quadruplicate binding experiments or quintuplicate cell growth experiments.

Results

Effect of VIP and Related Peptides on Cell Growth

Generally, the cell proliferation assay demonstrated that VIP and related peptides were potent stimulators of C6 cell proliferation (Fig. 1A). VIP exerted a concentration-dependent strong proliferative effect, by up to 69 ± 9%. This response was biphasic over a first concentration range from 10^{-15} to 10^{-13} *M* and a second range from 10^{-11} to 10^{-8} *M*, whereas no significant stimulation was observed at

the concentration of 10^{-12} *M*. Though slightly less potent than VIP, PACAP-27 also induced a significant dose-dependent stimulation (up to 68 ± 10%) of cell growth, starting at the lowest concentrations. This response was monophasic: Limited effects were observed for low concentrations ranging from 1 f*M* to 1 p*M* concentrations, whereas a dose-dependent increased cell proliferation (with a half-maximal effect in the nanomolar range) was observed for concentrations ranging from 1 p*M* to 10 n*M*. On the other hand, addition of glucagon induced limited though significant cell growth inhibition at the lowest concentrations (–15 ± 4% at 10^{-14} *M*); poor effects on cell proliferation were then observed for higher values, except for the highest doses tested (~50% stimulation for a 10 n*M* treatment). The VIP analog PHI alone also stimulated cell growth poorly (Fig. 1A), whereas in combination with VIP (10^{-13} *M*), this peptide induced a strong and sensitive stimulation of cell proliferation for all concentrations tested, with a maximum increase of 124 ± 33% occurring at 10^{-10} *M*. These effects were always higher than those observed with either VIP or PHI alone (Fig. 1B).

Effects of antagonists on cell growth were also studied for concentrations starting from 0.1 p*M*. To facilitate reading, antagonists [N-Ac-Tyr^1, D-Phe^2]-GRF (1–29) amide, [N-Ac-Tyr^1, D-Arg^2]-GRF (1–29)-NH_2, neurotensin (6–11)-VIP (7–28) will be designated, respectively, as VIP antagonist, GRF antagonist, and neurotensin-VIP, for the purpose of this article only.

All of the antagonists tested generally induced a dose-dependent cell proliferation increase, compared with the growth of untreated cells (Fig. 2A). A significant stimulation of cell growth was observed for concentrations starting from 10^{-13} *M* for the VIP antagonist and neurotensin-VIP and from 10^{-11} *M* for the GRF antagonist.

On the other hand, the cotreatment of 10^{-9} *M* VIP with increasing concentrations of each of the three antagonists generally resulted in a significant decrease of the proliferation induced by 10^{-9} *M* VIP alone (corresponding to ~50% increase of cell growth), the extent of which varied depending on the antagonist utilized (Fig. 2B). A combination of 1 n*M* VIP, together with neurotensin-VIP or the GRF antagonist, did not cause any significant stimulation of cell proliferation for concentrations ranging from 1 f*M* to 0.1 n*M*. Then a weak stimulation of the proliferation was observed for higher concentrations (1 and 10 n*M*) of these antagonists (up to ~25%), together with 1 n*M* VIP, however, to a much lower extent than that observed with 1 n*M* VIP alone (50% stimulation, as indicated above). In contrast, the VIP antagonist never readily suppressed the 1 n*M* VIP-induced stimulation of cell proliferation, despite the concentration tested. However, this antagonist utilized together with 1 n*M* VIP triggered a reduced stimulation of cell proliferation (with a maximal increase of 26 ± 6% at 10^{-10} *M*), in comparison with 1 n*M* VIP acting alone (50% stimulation).

Binding Studies

Data from ^{125}I-labeled VIP binding displacement experiments (Fig. 3A) suggested the expression of two different binding sites for VIP in C6 cells: very high-affinity binding sites (46% of the total binding sites [IC_{50}: $1.5 \times 10^{-14} \pm 0.5 \times 10^{-14}$ *M*]) and high-affinity sites (IC_{50}: $1.7 \times 10^{-10} \pm 0.3 \times 10^{-10}$ *M*). The curve corresponding to ^{125}I-labeled VIP displacement by increasing concentrations of PACAP fitted a one-site equation well and indicated a high-affinity interaction of PACAP-27 on ^{125}I-labeled VIP binding sites in C6 cells (IC_{50}: $1.1 \times 10^{-8} \pm 0.1 \times 10^{-8}$ *M* for PACAP-27). No displacement of ^{125}I-labeled VIP binding by PHI was observed.

Experiments of competitive displacement of ^{125}I-labeled PACAP binding (Fig. 3B) revealed that unlabeled VIP and PACAP-27, but not PHI, were able to interact with ^{125}I-labeled PACAP binding sites, with a similar high affinity (IC_{50}: $1.2 \times 10^{-9} \pm 0.1 \times 10^{-9}$ *M* for VIP and $7.1 \times 10^{-9} \pm 0.1 \times 10^{-9}$ *M* for PACAP-27). The binding of ^{125}I-labeled PACAP on C6 cells was entirely displaced by VIP.

Data from ^{125}I-labeled VIP binding displacement experiments by the antagonists (Fig. 4) also fitted a one-site equation well. However, all three antagonists interacted only with the portion of the ^{125}I-labeled VIP binding displacement curve corresponding to very high-affinity binding sites. In fact, very low IC_{50} values were observed for all three antagonists: $IC_{50} = 1.5 \times 10^{-13} \pm 0.3 \times 10^{-13}$ *M* for neurotensin-VIP; $IC_{50} = 1.2 \times 10^{-13} \pm 0.2 \times 10^{-13}$ *M* for the GRF antagonist; and $IC_{50} = 1.7 \times 10^{-13} \pm 0.4 \times 10^{-13}$ *M* for the VIP antagonist.

Discussion

The data reported here demonstrate that VIP, PHI, PACAP-27, and glucagon efficiently modulated the proliferation of rat glioblastoma C6 cells, each peptide (or peptide combination) displaying a distinct behavior in this regard. A strong stimulation was obtained with VIP and PACAP-27, but only VIP

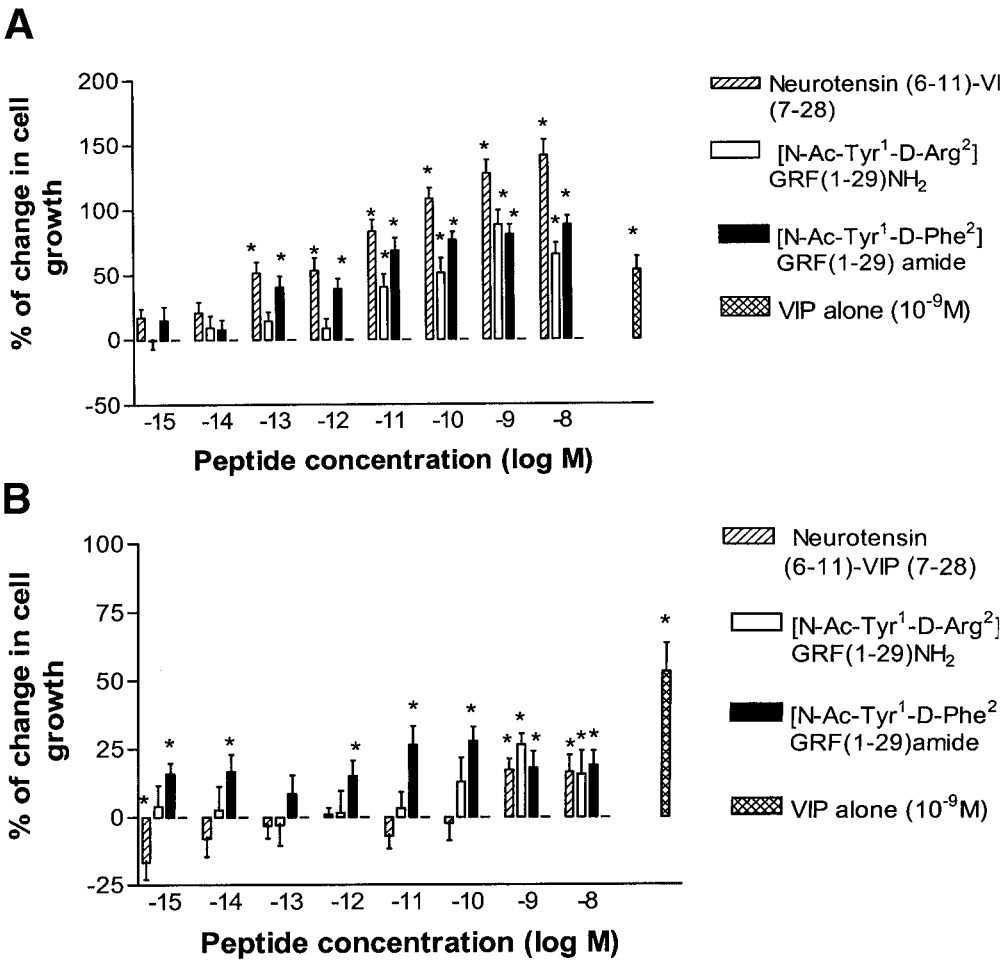

Fig. 2. Effect of three exogenous VIP receptor antagonists on the proliferation of C6 cells in culture: neurotensin-VIP; GRF antagonist; and VIP antagonist. Cell growth was expressed as the percentage of variation of cell number after treatment with VIP receptor antagonists, compared to cell number observed without treatment (control) (0%) **(A)** (*) $p < 0.05$ vs untreated cells), or the percentage of variation of cell number after treatment with VIP receptor antagonists together with VIP 10^{-9} *M*, compared to cell number observed without treatment (control) (0%) **(B)** (*) $p < 0.05$ vs untreated cells). Data are the mean ± S.E.M. of three independent experiments, each performed in quintuplicate ($n = 15$).

appeared particularly potent at very low concentrations, in the femtomolar range. Glucagon, on the other hand, inhibited proliferation at low concentrations while it stimulated proliferation only for higher doses (10^{-9}–10^{-8} *M*). The VIP analog PHI, acting alone, exerted no significant or very modest effects in the range of concentrations utilized but appeared to be efficient to potentialize the action of 0.1 p*M* VIP on cell proliferation. This observation indicates that distinct binding sites interacting selectively with PHI, but not with VIP, might mediate the potentialization exerted by PHI on VIP-induced stimulation of cell proliferation. However, the action of PHI acting alone on its selective binding sites might not be able to induce any growth stimulation effect.

A particularly intriguing question was the biphasic proliferative response to VIP for concentrations ranging from 10^{-15} to 10^{-13} *M* and from 10^{-11} to 10^{-8} *M*, whereas 10^{-12} *M* VIP triggered poor effects. Such data indicate that different subtypes of receptors might mediate this complex effect of VIP. Biphasic responses to peptides of the VIP family have been reported previously in other systems. For example, VIP at 50 p*M* stimulated proliferation of cultured retinal-pigmented epithelium cells, whereas higher concentrations inhibited proliferation (Koh, 1991). In addition, biphasic action of VIP on cell growth

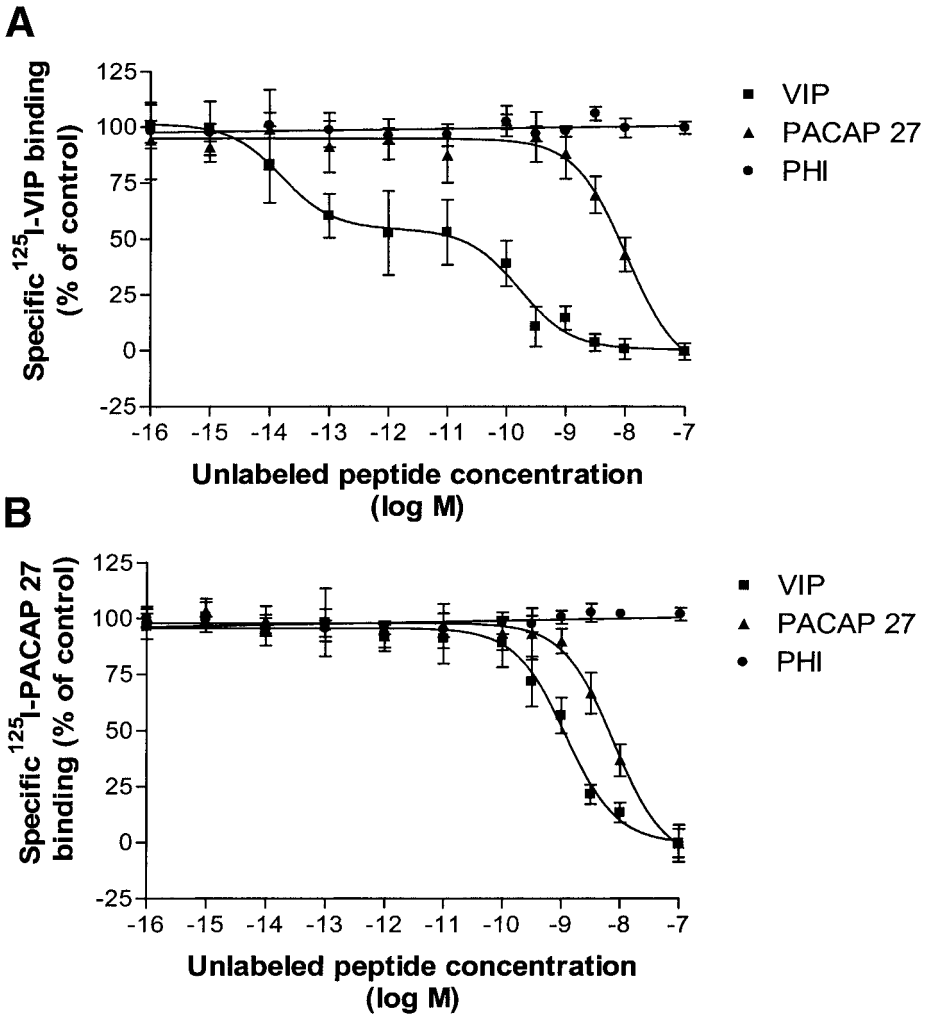

Fig. 3. Competitive displacement of ^{125}I-labeled VIP binding **(A)** and ^{125}I-labeled PACAP-27 binding **(B)** by unlabeled peptides: VIP, PACAP-27, and PHI. Data represent the percentage of specific binding compared to control (100%, in the absence of any competitor peptide). Data are the mean ± S.E.M. of three independent experiments, each performed in quadruplicate. Total specific binding: 493 cpm (VIP); 533 cpm (PACAP-27).

has been reported for Lo-Vo colonic carcinoma cells (Yu et al., 1992) and T98G glioblastoma cells (Vertongen et al., 1996). A similar effect was also described by Lelièvre et al. (1998b) on Neuro2a neuroblastoma cells after stimulation by PACAP, and by Chneiweiss et al. (1986) in the case of stimulation exerted by VIP on adenylate cyclase in glial cells. Furthermore, VIP appears to be inhibitory or stimulatory in function of the model studied. It stimulated cell proliferation in mouse astroglial cells (Brenneman et al., 1990), whereas it inhibited proliferation in others, like the human neuroblastoma IMR 32 (O'Dorisio et al., 1992) and the T98G glioblastoma (Vertongen et al., 1996). A lipophilic VIP analog, stearyl-Nleu(17)-neurotensin (6–11)-VIP (7–28), enhanced the antiproliferative effect of chemotherapeutic agents, such as doxorubicin, cisplatine, and vinorelbine, on advanced solid tumors, such as nonsmall cell lung carcinoma, colon carcinoma, or prostate carcinoma (Gelber et al., 2001).

In our studies, proliferative effects obtained with PACAP-27 treatments were quite modest for low concentrations of the peptide, ranging from 10^{-15} to 10^{-13} *M*. Then a dose-dependent increased cell growth was observed for higher concentrations for which PACAP-27 was about as potent as VIP to stimulate cell proliferation. Hence, PACAP-27 does not reproduce the effect of extremely low concentrations of

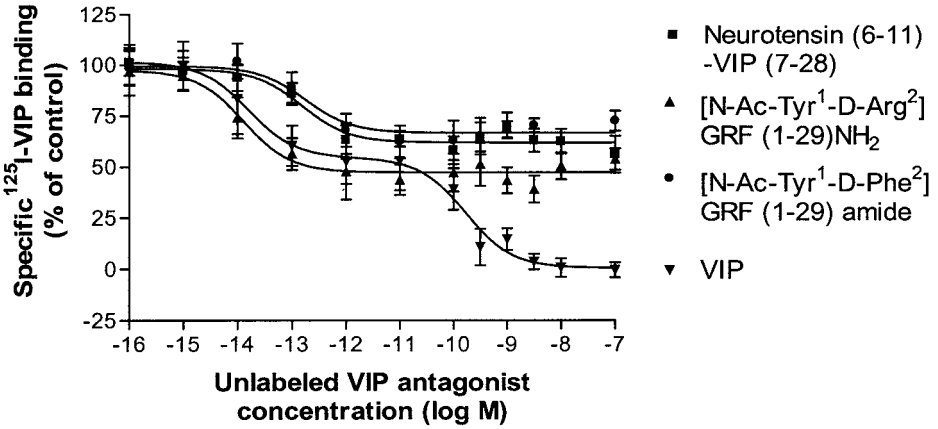

Fig. 4. Competitive displacement of ^{125}I-labeled VIP binding by unlabeled VIP antagonists, neurotensin-VIP, GRF antagonist, and VIP antagonist. Data represent the percentage of specific binding compared to control (100%, in the absence of any competitor peptide). Data are the mean ± S.E.M. of three independent experiments, each performed in quadruplicate. Total specific binding: 493 cpm (VIP).

VIP on cell proliferation, indicating that this VIP analog might not be able to activate the subset of VIP binding sites involved in this effect. Effects of PACAP-27 have been demonstrated previously by Waschek et al (2000) on the proliferation of the mouse neuroblastoma tumor cell line Neuro2a at subnanomolar doses (>10% increase in thymidine incorporation occurring at 10^{-10} *M*), whereas PACAP was shown by Vertongen et al (1996) to inhibit human glioblastoma T98G cell proliferation.

Of particular interest was the significant antiproliferative action of glucagon, observed only for concentrations lower than 10^{-12} *M*. Such effects have not been mentioned before for this peptide. However it has been shown by Pavelic and Pavelic (1980) that glucagon retards the growth of mammary aplasic carcinoma in vivo and prolongs the mean survival time of the animals treated. It should thus be interesting to analyze in C6 cells the potential expression of glucagon receptors that might mediate the modulatory action of glucagon on VIP-induced cell proliferation.

Because exposure to VIP increased cell proliferation, we also examined in this study the effects of treatments with antagonists on the growth of C6 cells, alone or together with a given 1 n*M* VIP concentration that triggered a maximal effect on proliferation after a 3-d treatment. Exposure of C6 cells to neurotensin-VIP, GRF antagonist, or VIP antagonist acting alone; respectively, resulted in a dose-dependent increase of cell proliferation. The order of efficiency of these peptide derivatives to stimulate cell growth was neurotensin-VIP > VIP antagonist > GRF antagonist.

In the presence of 10^{-9} *M* VIP, all three antagonists very significantly reduced cell growth compared to the effects obtained with 1 n*M* VIP alone, despite the antagonist concentration used. For concentrations ranging from 10^{-15} *M* to 10^{-11} *M*, the neurotensin-VIP and the GRF antagonist completely blocked the action of VIP, thus resuming the same cell growth as the control cells that received no peptide treatment at all. Treatment with the lowest concentrations of neurotensin-VIP even resulted in a reduction in cell number, compared to untreated control cells, with a maximal reduction (about –20%) occurring for the 10^{-15} *M* antagonist. The order of efficiency of these peptide derivatives to inhibit cell growth stimulated by 1 n*M* VIP was neurotensin-VIP > GRF antagonist > VIP antagonist.

Interestingly, the biphasic dose-response curve observed by cotreating cells with the VIP antagonist, together with 1 n*M* VIP, was very close to the shape of the dose-response curve obtained with increasing concentrations of VIP alone, except that the amplitude of the response was globally reduced.

Such an inhibition of VIP effect by these molecules has been described previously (Gozes et al, 1995). Acute administration of neurotensin-VIP resulted in blockade of VIP-mediated potentiation of sexual behavior in the adult rodent. During gestation, severe microcephaly was induced by acute administration of neurotensin-VIP to pregnant mice. This antagonist appears to be a competitive blocker

for both VIP-mediated increase of cAMP production or VIP-associated maintenance of neuronal survival in spinal cord cultures (Gozes et al., 1991). The VIP antagonist was shown to selectively inhibit both VIP and GRF-stimulated adenylate cyclase activities in rat pancreatic plasma membranes (Waelbroeck et al., 1985; Robberecht et al., 1986). The GRF antagonist was able to inhibit the GRF-stimulated adenylate cyclase in adenopituitary membranes (Robberecht et al., 1986).

The observations of the differential effects of peptides (or peptide combinations) considered in the present studies suggested the hypothesis that multiple binding sites for these peptides were expressed in C6 cells, which led us to characterize the pharmacological profiles of the ^{125}I-labeled VIP and ^{125}I-labeled PACAP-27 binding sites expressed in C6 cells. These parameters have not been characterized so far on C6 cells. Data from experiments of inhibition of the binding of ^{125}I-labeled VIP or ^{125}I-labeled PACAP-27 radiotracers by unlabeled VIP, PACAP-27, or PHI were consistent with the expression of two different VIP and PACAP binding species:

- VIP-selective very high-affinity binding sites with an uncommonly low IC_{50} value in the femtomolar range (estimated to be 48% of the total VIP binding species). This fraction of sites displays a 10^5-fold lower IC_{50} value for VIP than for PACAP.
- Bivalent high-affinity VIP/PACAP binding sites with similar IC_{50} values for both peptides are in the nanomolar range.

In all experiments conducted, PHI never inhibited the binding of 125I-VIP and ^{125}I-labeled PACAP-27, clearly indicating that potential effects of this peptide on C6 cells could not result in the interaction of this peptide with the VIP and PACAP-27 binding species expressed in this cell line.

Hence, C6 cells express binding species that could correspond to the VIP/PACAP receptors, the so-called VPAC1 and VPAC2 molecular species (using the International Union of Pharmacology nomenclature), which were extensively characterized, in terms of sequence, pharmacological profile, tissue expression, and associated signaling pathways in several tissues and cell lines. The expression of VPAC2 receptors involved in antiproliferative effects of VIP or PACAP has been demonstrated in glial tumors and in the human glioblastoma cell line T98G (Vertongen et al., 1996). However, PACAP-preferring high-affinity binding sites of the PAC1 subtype, which binds PACAP more exclusively with modest affinity for VIP and practically no interaction with PHI, were demonstrated to be expressed in surgical specimens of human glioblastomas and in several types of gliomas (Robberecht et al., 1994; Vertongen et al., 1996).

However, the most uncommon data obtained in the present studies was the finding of VIP-selective very high-affinity binding sites. The expression of VIP-selective high-affinity binding sites in glial cells (Brenneman et al., 1987; Gozes et al., 1991), in the human glioma cell line U-343 Mgcl 2:6 (Nielsen et al., 1990), and in the human glioblastoma XF-498L (Fabry et al., 2000) has been reported previously, and it has been demonstrated that the neuroprotective effects of VIP or PACAP could be observed for very low concentrations, close to the femtomolar range (Kong et al., 1999; Offen et al., 2000). However, data from the present studies are, as far as we know, an unprecedented characterization of VIP-selective binding sites with such a very high affinity, in the femtomolar range. The affinity of these very high-affinity sites for VIP is about 10,000-fold greater than that of the "traditional" high-affinity receptors VPAC1 or VPAC2, suggesting that VIP could act in C6 cells at extremely low concentrations. As a matter of fact, the remarkably high affinity of these sites, strongly suggests that they are the candidate receptors to induce the increased C6 cell proliferation observed in the present study for concentrations lower than 10^{-12} *M* VIP. The interaction of VIP with traditional high-affinity binding sites, corresponding to VPAC1 or VPAC2 receptors, could account for the VIP-induced growth stimulation observed for concentrations higher than 10^{-12} *M*. Expression of both subtypes of binding sites in the C6 cell line thus explains the biphasic proliferation response observed when these cells are treated with increasing concentrations of VIP.

Another prominent property of the very high-affinity VIP binding sites expressed in C6 cells is their selective interaction with the three antagonists evaluated in our studies. In fact, these components only partially inhibited the ^{125}I-labeled VIP interaction in C6 cells, affecting only the portion of the displacement curve corresponding to the very high-affinity VIP binding sites. The IC_{50} values calculated for these antagonists in the femtomolar range confirm that these molecules behave like very selective and very high-affinity ligands for a fraction of the ^{125}I-labeled VIP binding sites expressed in C6 cells. It has been long reported that GRF, besides its high-affinity interaction with pituitary GRF receptors (Robberecht et al., 1985), also interacted with VIP

receptors (Laburthe et al., 1983; Waelbroeck et al., 1985), probably with a higher affinity for VPAC1 than for VPAC2 receptors (Robberecht et al., 1996). Conversely, GRF had only a negligible interaction with the rat PACAP-preferring receptors (Gourlet et al., 1997). Primary reports on the VIP antagonist considered in this study, or on neurotensin-VIP, indicated that these components generally behave like low-affinity ligands (in the micromolar range) of the VIP binding sites of peripheral cells from different tissue origins, such as small-cell lung carcinomas or human neuroblastomas (Moody et al., 1993; Lilling et al., 1994). Other investigators have found that neurotensin-VIP inhibited ^{125}I-labeled VIP binding to cells from cerebral cortex, hippocampus, or spinal cord with K_i values in the picomolar or nanomolar range, respectively, for high- and low-affinity binding sites. The antagonizing effects of neurotensin-VIP occurred on neuronal cell with an IC_{50} value of 30 p*M* (Gozes et al., 1991). It is worth noting that antiproliferative effects of neurotensin-VIP in vitro and in vivo (on xenografts in nude mice) have also been related to the antagonist properties of this hybrid peptide toward PACAP-selective receptor PAC1 (Sharma et al., 2001). Taken together, these data suggest that very high-affinity VIP binding sites such as those observed in this study might not be frequent in peripheral tissues but might be represented much more in the CNS, possibly in glial cells, mediating typical effects of extremely low concentrations of VIP or other related peptides in this tissue. The three antagonists considered in the present study appear to behave like very selective ligands, able to counteract effects of very low concentrations of VIP on cell growth, possibly mediated through the unusual very high-affinity binding sites. However, higher concentrations of these antagonists also exert potent agonist properties on C6 cell proliferation when acting alone. An explanation for this peculiar phenomenon could be that VIP and the antagonists mutually compete with each other for their interaction with—at least partially—overlapping binding domains, thus embedding the efficient activation of their common receptors. A similar observation was reported previously by our group in the colonic carcinoma cell line SW403 treated by VIP, PACAP, or both peptides (Lelièvre et al., 1998a): We demonstrated that combined treatments with both agonists were less efficient to regulate cGMP production than treatments with individual peptides. Such data indicate that combining two potent peptide agonists could result in pseudoantagonist effects, possibly through a competition of both peptides at the level of common binding sites, resulting in a decreased efficacy of the combined peptides to activate the receptors.

In conclusion, we demonstrated that VIP and the related neuropeptide PACAP-27 stimulated the proliferation of C6 rat glioblastoma cells, whereas antagonists inhibited the growth effects of VIP at extremely low concentrations, probably acting through an unusual subclass of very high-affinity VIP binding sites, displaying a K_d value in the femtomolar range. Hence, we propose that these antagonists, neurotensin-VIP and the two GRF derivatives, should be considered as invaluable tools to further characterize the pharmacological, molecular, and functional properties of the very high-affinity selective VIP receptors expressed in the C6 glioblastoma cell line.

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