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VIP as a Cell-Growth and Differentiation Neuromodulator Role in Neurodevelopment

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Abstract

In addition to its commonly recognized status as a neuromodulator of virtually all vital functions, including neurobiological, the neuropeptide VIP plays a role in the control of cell growth and differentiation and of neuronal survival. Through these actions, VIP, whose impact appears early in ontogeny, may possess developmental functions. VIP can be stimulatory or inhibitory on cell growth in function of the model considered. The growth regulatory actions of VIP, which are often independent of cAMP, are most likely significant when mitogenic or trophic factors, eventually released by nontarget cells, are simultaneously present in the extracellular medium. The intracellular mechanisms that mediate these actions of VIP may involve different transduction cascades triggered by subsets of VIP binding sites that may coexist in the same tissue.

Index Entries: Vasoactive intestinal polypeptide (VIP); neurodevelopment; VIP receptors; cell growth; differentiation; signaling pathways.

Introduction

In 1970, the 28 amino-acid Vasoactive Intestinal Polypeptide (VIP) was isolated by Said and Mutt from intestinal extracts, on the basis of its potent vasodilator activity. The peptide is a structural analog of secretin, glucagon, GRF (growth-hormone-releasing factor), and of the more recently discovered peptides PHM/PHI (peptide having carboxy terminal methionine/isoleucine), helodermin, and PACAP (pituitary adenylate cyclase activating polypeptide). Initially considered like a potential gastrointestinal hormone, it appeared that circulating VIP level, in the range of the picomolar, was far below the concentrations necessary to trigger its known physiological activities, and was not significantly increased in given physiological conditions, such as digestion. Exceptional systemic actions of VIP can be observed in the syn-

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drome of chronic watery diarrhea of Werner-Morrison (pancreatic cholerae), owing to the hypersecretion of the peptide by tumors known as VIPomas usually arising from pancreatic islet cells or neural tissue. In 1976, the presence of immunoreactive VIP was demonstrated in brain and other nervous tissues. Today, VIP is commonly recognized as a neuropeptide with wide distribution in the central and peripheral nervous systems and a broad spectrum of biological actions. The peptide acting as a neurotransmitter or neuromodulator participates in a multifactorial regulation of vital functions as diverse as digestion, breathing, reproduction, immunomodulation, or again in the control of blood flow, brain metabolism, and release of pituitary neurohormones (Said and Mutt, 1970; Said and Rosenberg, 1976; Said, 1986, 1991, 1993).

The neurobiological actions of VIP have been studied extensively and reported in several excellent reviews (Rostene, 1984; Gozes and Brenneman, 1989). In concert with other neurotransmitters, VIP can modulate electrical responses, since the peptide induces either excitatory or inhibitory potentials in preoptic, septal, and midbrain neurons. However, the most frequent response consists of a rapid depolarization with a large increase in membrane potential. Slow depolarization triggered by VIP in retinal horizontal cells appear independent of cAMP, suggesting that VIP receptors are not always linked to cAMP-mediated processes (Lasater et al., 1983). An increase in the frequency of calcium-dependent action potentials and modulation of voltage-sensitive conductances in response to VIP have been related to prolactin secretion in the pituitary (Hedlund et al., 1988). Interestingly, glial cells have also been shown to respond to VIP by changes in membrane potential (Evans and Villegas, 1988; Magistretti, 1988). Modulation of adrenergic responses and facilitatory effects on muscarinic excitation are also reported (Ferron et al., 1985; Kawatani et al., 1985). Acetylcholine activity may be potentiated through an increase by VIP of the affinity of the muscarinic receptor for the neuromediator (Lundberg et al., 1982). Part of the cholinergic innervation in the cortex is also VIPergic, suggesting the importance of the interactions between VIP and acetylcholine in the brain (Eckenstein and Baughman, 1984; Luine et al., 1984). Several substances are induced to be secreted by VIP in neural tissue. Intracerebroventricular application of the peptide stimulates the liberation of oxytocin and vasopressin (Bardrum et al., 1988). Calcium-dependent secretion of catecholamines has been observed in chromaffin cells (Misbahuddin et al., 1988). Modulation by VIP of the release of somatostatin in the cortex and hypothalamus has also been described (Tapia-Arancibia and Reichlin, 1985). Rhythmic circadian production of VIP in the suprachiasmatic nucleus (Alberts et al., 1990; Okamoto et al., 1991) appears to modulate the liberation of melatonin in the pineal where the peptide interacts with high affinity binding sites, which results in a stimulation of cAMP production and activation of serotonin N-acetyl transferase (NAT) activity (Meunier et al., 1991). Hypothalamic VIP released in the hypophyseal portal blood flow stimulates the release of prolactin and possibly of ACTH (Reichlin, 1988). It has been proposed that VIP released by lactotrope cells may control prolactin secretion in an autocrine fashion (Nagy et al., 1988). In addition to its neuroendocrine functions, the biological actions of VIP in the central nervous system (CNS) still being defined are: the control of the electrical activity of cortical and spinal cord neurons; the stimulation of glycogenolysis and glucose utilization in various brain areas, particularly in the cortex; hypnogenic and antidipsogenic effects (Magistretti et al., 1981; Said, 1986, 1991).

Association of an abnormal impact of VIP with human disease has also been proposed; in particular, lacking or deficient VIPergic innervation has been observed in colonic Hirshprung's disease or esophageal smooth-muscle achalasia, or around sweat glands, in patients with cystic fibrosis. Potential therapeutic applications of VIP are its utilization as a relaxant of tracheobronchial smooth muscle in the management of asthma, or as an inhibi-

tor of immunologic release of mediators. As a vasodilator, the neuropeptide may be used to promote local or regional blood flow or reduce hypertension. In the peripheral nervous system, VIP has been shown to participate in the regulation of pain transmission. Possible involvement of VIP in brain pathologies, including epilepsy, has also been proposed (Said, 1991). The limit in clinical applications of VIP is its rapid inactivation by target cells, through receptor-mediated endocytosis and subsequent degradation (Muller et al., 1985; Luis et al., 1986). Furthermore, hepatic clearance of circulating VIP (Misbin et al., 1982), enzymatic degradation (Hachisu et al., 1991), or possible inactivation by catalytic antibodies (Paul and Ebadi, 1993) suggest that the peptide should be more effective when delivered locally rather than through systemic blood flow.

Beside these "traditional" actions of VIP, more recent data demonstrate that VIP participates in the establishment or maintenance of the differentiated phenotype and in cell growth regulation in safe or tumoral tissues, hence indicating potential properties of the peptide as a developmental factor. A promising discovery was the demonstration that VIP acting at picomolar concentrations induced the release by glial cells of substances necessary for neuronal survival. This makes VIP a mediator of communication between neurons and glia, which is a key property for a factor influencing the function and development of neural tissue.

The aim of the present review is to present this growing array of evidence that assigns VIP the novel status of a developmental neuromodulator. This report, mainly focusing on data concerning neurodevelopmental roles of the peptide, will be enlarged to the corresponding effects in other tissues, in order to compare the modalities of its action in these different targets.

Distribution and Release of VIP

As previously reviewed (Rostene, 1984; Said, 1986; Reichlin, 1988; Gozes and Brenneman, 1989), immunofluorescence and radioimmunoassay techniques demonstrated the wide distribution of the VIPergic innervation in the central and peripheral nervous systems. The peptide, observed in neuronal cell bodies, axons, and dendrites, is concentrated in synaptosomes (presynaptic nerve terminals), where it can be released as a neurotransmitter. Data concerning the distribution of VIP in the CNS demonstrate that the highest densities of VIP-containing neurons occur in the hypothalamus (particularly the suprachiasmatic and paraventricular nuclei), around the median eminence and in the cerebral cortex, mainly in a distinct population of radially oriented, bipolar interneurons. At the peripheral level, immunoreactive VIP has been localized in the sympathetic ganglia, in the vagus, and in motor (sciatic) nerves. An intrinsic VIP innervation is supplied through VIPergic clusters in autonomic ganglia present in the vicinity of exocrine glands, epithelia, and nonvascular smooth muscles. Relatively important levels of VIP are detected in the gastrointestinal tract (except liver), lung, heart, genital organs, kidney, urinary bladder, spleen, skin, and lymphatics. Significant VIP concentrations are present in intraocular tissue (particularly retina), bones, thyroid, and pineal. Coexistence of VIP with other neurotransmitters is well documented. The neuropeptide coexists with acetylcholine in central and peripheral cholinergic neurons, with PHI/PHM in several tissues from which both peptides may be coreleased or with enkephalins and catecholamines in adrenal chromaffin granules. Autonomic ganglia and the enteric innervation contain VIPergic neurons that also express part or all of the following list of (poly)peptides: CCK, dynorphin, GRP, and enkephalins.

Distinct groups of cells, such as platelets, mast cells, skin MERCKEL cells, neutrophils, and retinal amacrine cells, appear to be able to synthesize and release VIP. Conditions of release of neuronal VIP have been studied using pharmacological and physiological stimuli, sometimes in the presence of pharmacological blockers, to help define its mechanism of liberation. Release of VIP induced by

electrical stimulation is unaffected by adrenergic or cholinergic muscarinic blockade, but abolished by hexamethonium in hypogastric or pelvic nerves. Tetrodotoxin blocked electrical field stimulation of VIP release in cerebral tissues, but also in ileum, trachea, and pancreas. Factors that raise intracellular cAMP, phorbol esters, retinoic acid, ascorbic acid, NGF, high potassium, veratridine, and nicotine stimulate VIP biosynthesis in neuroblastomas or endocrine cells such as pheochromocytomas and chromaffin cells. Conversely, dexamethasone decreased VIP production in human neuroendocrine cells (Beinfeld et al., 1988; Waschek and Eiden, 1988; Waschek et al., 1989; Georg et al., 1992; Yamaguchi et al., 1992).

Biosynthesis of VIP: Variable Molecular Forms

In 1983, Itoh and collaborators identified and sequenced a cDNA encoding the human VIP precursor and discovered that this molecule contained not only VIP, but also a VIP analog called PHM that was almost identical to the 27-amino-acid PHI expressed in other species (Tatemoto et al., 1984). These polypeptides differ through their carboxy terminal residue methionine for PHM and isoleucine for PHI. The human VIP/PHM gene was then isolated and assigned to region q24 on chromosome 6 (Gozes et al., 1986, 1987a).

These breakthroughs led to studies on developmental regulation of the VIP-gene expression in rat brain. The resulting data were of chief importance since the developmental patterns of VIP-gene expression suggested a differentiation factor role for VIP in the embryo and in the newborn animal. For instance, increasing VIP-mRNA and VIP levels were observed from birth to 16 d of age in the frontal and parietal cortex. In the hippocampus, the major peak of VIP-mRNA occurred at 8 d, whereas hypothalamic VIP-mRNA content was already elevated at birth. In all cases, the peaks of mRNA content were preceding appearance of immunoreactive peptide, indicating that other peptide products of the VIP gene may appear at an early stage. It is worth indicating that in these experiments, the VIP-mRNA of about 2000 bases is detected together with higher VIP-related RNAs forms of about 5000 and 7000 bases that represented 80% of total VIP RNAs at birth and gradually decreased to about 20% at 30 d of age. These observations point to the fact that besides transcriptional regulations of the VIP gene, an additional control operating at the RNA processing level should allow fine tuning of VIP levels during brain maturation (Gozes et al., 1987b, 1988; Gozes, 1988; Gozes and Brenneman, 1989). In a recent report, it was demonstrated that VIP mRNA was not evident in the rat CNS until birth, whereas VIP receptors were abundantly expressed. This led to the proposal that either a yet-unidentified VIP-related component, VIP originating from elsewhere in the prenatal body, or extraembryonic VIP of placental or maternal origin, may act on prenatal VIP receptors to regulate ontogenic events in the brain (Hill et al., 1994).

In the peripheral nervous system (PNS), peaking VIP-mRNA levels are observed, particularly in the embryonal intestine in 16-d-old rat embryos, coinciding with establishment of the fetal circulatory system. Still high at birth, the VIP-mRNA content in the rat intestine decreases somewhat postnatally, then resumes higher values in adults in correlation with weaning (Gozes, 1988). In the human fetus, VIP expression appeared between the 8th and 11th wk and increased until the 24th wk of gestation, first in the intestine, then in the stomach and pancreas (Zheng et al., 1992).

Hyper- and hypocorticism have been associated with modifications of postnatal immunoreactive VIP levels. The role of corticoids in the establishment of the developmental patterns of VIP was confirmed by the demonstration that corticosterone administration resulted in an enhanced expression of the VIP-mRNA in the developing hypothalamus, in agreement with increased peptide levels. Another argument for the importance of steroid hormones in the control of VIP gene activity is the elevation in VIP-

mRNA in the hypothalamus of lactating female rats, associated with an increased VIP-immunoreactivity in paraventricular nucleus neurons. This correlates VIP stimulatory effects on prolactin release. Hence, the impact of VIP in the control of lactation may be modulated by changes in circulating steroids that occur at that period (Gozes and Brenneman, 1989; Lam et al., 1990).

Biosynthesis of the VIP/PHM precursor was demonstrated to be increased by dibutyryl cAMP, forskolin, phorbol esters, or again by drugs like nicotin and Ba^{2+} that trigger activation of calcium-dependent mechanisms. These data indicate that inducible elements at the level of the VIP gene promoter interact with transcriptional factors activated by signaling pathways involving cAMP- and calcium-dependent protein kinases (Gozes, 1988; Waschek and Eiden, 1988; Gozes and Brenneman, 1989; Waschek et al., 1992). Several investigators undertook to decipher the structure of the human VIP gene promoter and identified several TATA boxes. Two segments demonstrated as being inducible by cAMP and TPA were located; the first, close to the cap site, appears less sensitive than the second, located much further, 4.0–4.6 kb upstream. Targeting expression of a reporter gene in transgenic mice using 5.2 kb human VIP gene 5' flanking sequences resulted in the expression of the reporter in the intestine but not in the CNS (Takahashi et al., 1988; Waschek et al., 1988, 1992). This indicates that VIP gene expression in the CNS may require other yet-unidentified segments or that human and mice VIP gene 5' flanking sequences are somewhat different.

Another compound demonstrated to be efficient in the induction of the VIP gene expression is the vitamin A derivative retinoic acid (RA), which plays important roles in differentiation and morphogenesis. Its action on VIP gene expression has been checked in vitro in human neuroblastoma cell lines, like NB-I or SK-N-SH. In all cases, RA also increased the expression of the VIP/PHM precursor mRNA and the production and release by cells of immunoreactive VIP. These data are of particular importance, since they suggest that induction of the VIP gene may contribute to the establishment of the developmental pattern in embryonic tissues submitted to the differentiating action of retinoids (Waschek et al., 1989; Georg et al., 1992).

As discussed earlier, transcriptional regulation as well as possible alternative splicing and/or polyadenylation of the VIP/PHM precursor mRNA probably participate in the regulation of the VIP gene expression. Beside VIP and PHM, the precursor protein appears to generate higher size VIP-immunoreactive material, including carboxy-terminal extended forms of VIP, probably owing to differential proteolytic processing at basic residues (Gafvelin et al., 1988; Fahrenkrug, 1992; Li et al., 1992). Shorter forms of VIP have also been isolated in the brain (Romualdi et al., 1992), possibly resulting from proteolytic cleavage of the 28-amino-acid peptide and raising the question of the eventual biological significance of degraded forms of VIP. This possibility is supported, for instance, by the demonstration that fragments like VIP (10–28) may have antagonistic properties in some systems (Bissonnette, et al., 1984; Brenneman and Eiden, 1986; Turner et al., 1986).

Regulation by VIP of the Release of Neurotrophic Factors

Since this typical property of VIP has been reviewed elsewhere (Brenneman et al., 1990; Gozes and Brenneman, 1993), we will summarize in this article the data that allow one to consider the peptide as a neurotropism regulator. One important model for such studies is mouse spinal cord neuron in culture. In this system, the neurons die after some days, this process being accelerated by electrical blockade and removal of conditioning substances released by cultured cells. This enhanced cell death is efficiently prevented by VIP acting at exceptionally low concentration, resulting in an increased survival of neurons incubated in the presence of the peptide. Concurrently, it has been observed that electrical blockade

inhibited endogenous VIP synthesis and release in this tissue (Brenneman et al., 1985, 1987; Brenneman and Eiden, 1986; Brenneman, 1988; Agoston et al., 1991). An interesting clue was that these effects of VIP on neuronal survival were only observed when non-neuronal cells were present in the culture. In addition, VIP-like immunoreactivity has been found in spinal cord cultures (Brenneman et al., 1987) and antisera to VIP aggravated neuronal cell death (Brenneman and Eiden, 1986). This array of data allows one to propose the idea that some VIP-releasing neurons may stimulate in a paracrine fashion glial, and eventually other non-neuronal cells, thus controlling the release of neurotrophic factors required for neuronal survival. As a matter of fact, glial cells express VIP receptors and the peptide increases cAMP production, glycogenolysis, and morphological changes in this tissue (Cholewinski and Wilkin, 1988; Magistretti, 1988). It was recently reported that VIP, in synergism with α-adrenergic agonists, augments at subnanomolar concentrations an intracellular calcium signal in some type I astrocytes from rat cerebral cortex (Fatatis et al., 1994). Subnanomolar concentrations of VIP were shown to cause the nuclear translocation of protein kinase C, with no increase of the intracellular cAMP levels, in neonatal rat cortical astrocytes (Olah et al., 1994). Furthermore, VIP stimulates the release of interleukin-1 and protease nexin-1 from astrocytes, two polypeptides that have been shown to be associated with neuronal survival (Brenneman et al., 1992). Comparison of the proteins released from nonstimulated glial cells to the electrophoretic pattern of proteins released from VIP-stimulated astroglia in culture reveals several polypeptides with potential neurotrophic properties. Through sequential biochemical procedures, a novel protein released by glial cells on stimulation by VIP has been isolated and called Activity Dependent Neurotrophic Factor (ADNF). This glycine-enriched protein acts at very low concentration and induces optimal survival of electrically blocked neurons at 0.1 n*M* (Gozes and Brenneman, 1993).

Regulation by VIP of Cell Growth and Differentiation

The idea that VIP plays a role in the control of proliferation in tumoral and nontumoral cells is supported by several lines of evidence, summarized in Table 1 for neural and glial cells and Table 2 for cells of other origins; all have been demonstrated to express high affinity VIP receptors. The data point to the fact that VIP action in this regard does not obey simple schemes, since the peptide appears to be inhibitory or stimulatory in function of the cell type studied. These actions of VIP are generally associated with a transient increase of cAMP levels in most of the target tissues considered. Several comments concerning the studies presented in the tables are worth adding, since they may indicate future interesting directions of investigation. All the neuroblastoma considered express together VIP precursor mRNA and VIP receptors, and most of them also produce VIP immunoreactive material. This suggests that autocrine and/or paracrine actions of proVIP derivatives may take place during the cancerous transformation of tissues of neuronal origin. The neuropeptide stimulates cell proliferation in neuroblastoma and in sympathetic neuroblasts, but in others, like the human cancerous cell line NB-OK1, proliferation is inhibited by VIP, with a parallel development of phenotypic features of differentiated neurons, such as neurite outgrowth. Some observations of promotion of differentiation by VIP have been reported in human colon carcinoma cells whose proliferation is inhibited by the peptide (Hoosein et al., 1989). This indicates that inhibition of cell growth by VIP may be associated with development of differentiation processes in the corresponding tissues. Most of the direct effects of VIP on mitogenesis and cell proliferation were readily observed in the presence of relatively elevated doses of the peptide, beyond the K_d values of the corresponding VIP binding sites. One interesting model cell line is Lo Vo, established from a human colonic adenocarcinoma,

Table 1
Effects of VIP on Cell Mitogenesis and/or Proliferation in Neuronal and Glial Cells[a]

Cell type	Action of VIP on growth and/or mitogenesis	VIP precursor mRNA	Production of immunoreactive VIP	Associated cellular events	References
Human neuroblastoma IMR 32	Inhibition	+	+	cAMP ↑ differentiation	O'Dorisio et al., 1992
Human neuroblastoma NB-OK-1	Inhibition	+	+	cAMP ↑ differentiation	Hoshino et al., 1993
Human neuroblastoma NMB	Stimulation	+	ND	cAMP ↑	Wollman et al., 1993
Subclones of human neuroblastoma SK-N-SH	Stimulation	+	+	cAMP ↑	Muller et al., 1989 and this review
Rat embryos sympathetic neuroblasts in culture	Stimulation	ND	ND	cAMP ↑	Pincus et al., 1990
Mouse astroglial cells	Stimulation	ND	ND	Release of neurotrophic factors	Brenneman et al., 1990

[a] ND = not determined.

Table 2
Effects of VIP on Cell Mitogenesis and/or Proliferation in Nonneuronal and Nonglial Cells[a]

Cell type	Action of VIP on growth and/or mitogenesis	VIP precursor mRNA	Production of immunoreactive VIP	Associated cellular events	References
Human nonsmall cell lung cancer	Stimulation	+	+	cAMP ↑[b]	Schaffer et al., 1987 Gozes et al., 1992
Human small cell lung cancer	Inhibition	–	–	cAMP ↑	Moody et al., 1992 Maruno and Said, 1992, 1993
Human pancreatic carcinoma Pu-Pan-1	Stimulation	+	+	cAMP ↑	Chen et al., 1992
Human colonic adenocarcinoma HT 29	Inhibition	ND[a]	ND	cAMP ↑	Gamet et al., 1992
Human colonic adenocarcinoma Lo Vo	<100 m*M*: Stimulation >100 m*M*: Inhibition	ND	ND	cAMP ↑, ODC ↑[c] cAMP (–), ODC ↓	Yu et al., 1992
Human gastric carcinoma cells	Inhibition	ND	ND	cAMP ↑, c-myc ↓	Kim et al., 1991
Human cultured keratinocytes	Stimulation	ND	ND	cAMP ↑	Haegerstrand et al., 1989 Takahashi et al., 1993
Human B-cells	Stimulation	–	–	cAMP (–), IgA ↑	Ishioka et al., 1992
Murine T-cells ConA stimulated	Inhibition	–	–	cAMP (–), IL_2 ↓[d]	Ottaway, 1987 Boudard and Bastide, 1991
Smooth muscle cells	Inhibition	ND	ND	cAMP ↑	Hultgardh-Nilsson et al., 1988
Mouse fibroblast 3T3 insulin-stimulated	Stimulation	ND	ND	cAMP ↑	Zurier et al., 1988

[a] ND = not determined.
[b] cAMP (–) = no stimulation of intracellular cAMP production.
[c] ODC = ornithine decarboxylase.
[d] IL_2 = interleukin 2.

that responds differentially to VIP, in function of the concentration of the peptide in the assay; for concentrations <100 n*M*, VIP stimulates intracellular cAMP production and concurrent with cell growth and ornithine decarboxylase (ODC) activity, the latter enzyme being a key component in the polyamine metabolism. For concentrations over 100 n*M*, no further increase in the intracellular cAMP levels is observed, whereas VIP appears to inhibit cell growth and ODC activity as well. The data indicate that in Lo Vo cells, VIP triggers at least two distinct signaling cascades that participate in opposite controls of cell proliferation, as a function of the concentration of peptide utilized. The cascade leading to an increased cell growth appears to be related to intracellular cAMP elevation, whereas inhibition of proliferation may be mediated by a different mechanism. Hence, such a model cell line is of particular interest to investigate toward the differential transduction mechanisms that allow VIP to induce these opposite cellular responses. The same study also points out the role of polyamines that have been demonstrated to be essential for cell growth, but whose mechanisms of action are still poorly understood. The enzyme ODC catalyzes the first step of polyamine production from L-ornithine and it has been shown that activation of ODC is necessary to support the proliferative process in another adenocarcinoma cell line called HT29 (Gamet et al., 1991). This was brought together with the behavior of VIP-treated colonic adenocarcinoma cell lines whose proliferation is inhibited by the peptide while they progressively acquire an enterocyte-like phenotype. However, we demonstrated that VIP never stimulated cell growth in HT29 cells, but even caused a slight inhibition of proliferation (Fig. 1A).

A general comment that can be formulated concerning actions of VIP on cell growth concerns the experimental conditions that can be extremely divergent from one study to another, especially concerning the presence or absence of fetal calf serum (FCS) in culture medium, the cell density at the onset of the treatments, or

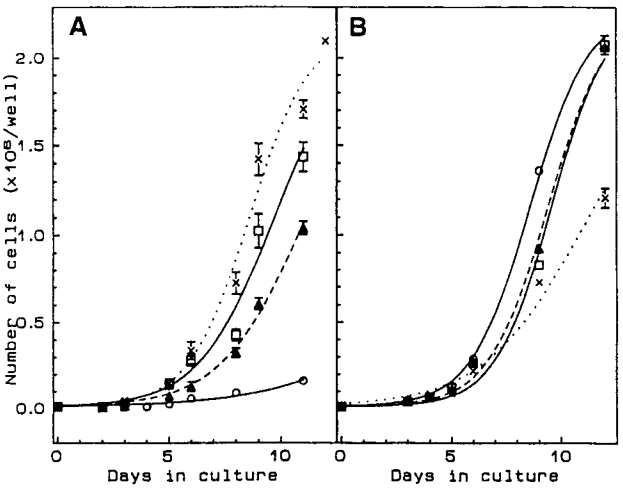

Fig. 1. Time courses of proliferation of **(A)** HT29, human colonic adenocarcinoma cells and **(B)** SH-IN, human clonal neuroblastoma cells in culture. Ten thousand cells were seeded in precoated 24-well culture plates and cultured in DMEM supplemented with 10% FCS and antibiotics. After 1 d in culture, drugs were added daily: VIP $10^{-8}M$ (□——), the adenosine antagonist XAC $10^{-6}M$ (▲---), VIP $10^{-8}M$ + XAC $10^{-6}M$ (○——), or no treatment: control cells (x ...). Culture medium was replaced every 3 d and cell number was evaluated in a counting chamber, after harvesting of the cells in trypsin/EDTA solution. Curves were fit by nonlinear regression analysis using the program GRAPHPAD.

again, the use of coated or uncoated culture plates. To circumvent these experimental discrepancies, we undertook to compare VIP action on cell proliferation in the human neuroblastoma SH-IN cells (a subclone of the SK-N-SH ganglionic cell line) and in the human colonic adenocarcinoma cells HT29, using strictly identical experimental conditions: Ten thousand cells were seeded in 24-well culture dishes precoated with HT29 extracellular matrix in culture medium containing 10% FCS. The action of 10 n*M* VIP resulted in a significant stimulation of cell proliferation in the neuroblastoma, whereas a slight inhibition of growth was observed in the colonic cancer cells (Fig. 1A,B). In these experiments, we also looked at the effects of an adenosine antagonist (xanthine amine congener: XAC) that competitively blocks extracellular effects of adenosine at the level of the nucleoside cell sur-

face receptors. Adenosine is released in the extracellular medium by virtually all cell types; the nucleoside is known to interact with two distinct extracellular receptors, named A_1 and A_2, respectively, coupled negatively and positively with adenylate-cyclase. The actions of adenosine acting as an extracellular messenger have been extensively documented; in particular, the nucleoside participates as a neurotransmitter in the regulation of neurobiological functions (Ohisalo, 1987; Stone, 1991) and modulates cell growth in epthelial (Tey et al., 1992) and glial tissues (Rathbone et al., 1992). Several adenosine analogs, agonists, or antagonists, interacting specifically with A_1 or A_2 receptors, have been developed and allow the study of differential effects of adenosine on these two binding subsites. The antagonist XAC blocks extracellular adenosine action at the level of the A_1 receptors negatively coupled to adenylate-cyclase (Cooper and Londos, 1988). We effectively demonstrated that XAC action in HT29 cells resulted in a significant elevation of the intracellular cAMP levels. Moreover, treatment of HT29 cells in the presence of XAC for several days in culture caused important cell growth inhibition, as illustrated in Fig. 1A. In our experiments, the action of XAC also triggered the development of enterocytic features in HT29 cells such as the appearance of junctional complexes and microvilli that are typical of cells of epithelial origin. These phenotypic modifications started to be obvious 3–6 d after the onset of treatment (manuscript in preparation). The actions of VIP and XAC on cell growth were clearly additive (Fig. 1A); this indicated that although both compounds trigger elevation of cAMP production, their subsequent signaling pathways may be at least partially different. We repeated in strictly identical conditions the experiments conducted in HT29 cells, in the human neuroblastoma SH-IN clonal cell line that express VIP receptors and in which the peptide efficiently stimulates cAMP production (Muller et al., 1989). We observed that in this cell line, XAC and VIP were equipotent in stimulating cell growth and that the combined actions of both compounds were poorly additive, suggesting that their effects are mediated in this case through a common mechanism (Fig. 1B). These data demonstrate that VIP and XAC actions result in a stimulation or in an inhibition of cell growth in the function of the model studied, and this in absolutely identical experimental conditions. These opposite actions are both related to stimulation of cAMP production by VIP and XAC. It is worth noting that in our experiments, no actions of VIP or XAC on cell growth were observed in absence of serum in both models tested. Another observation is that in our experiments, VIP acting alone is more efficient in stimulating than in inhibiting cell growth; however, it potentiates efficiently the inhibition but not the stimulation of cell growth induced by XAC.

The statement that stimulation and inhibition of cell growth by VIP are triggered by at least partially distinct signaling cascades is supported by several lines of evidence discussed in the present article. The nature of these transduction pathways still remain to be explored in detail and the cellular models proposed here should be useful for such investigation. The studies on adenosine action undertaken in our laboratory also indicate that the extracellular impact of this ubiquitous nucleotide may complement the action of VIP in the control of cell growth and differentiation. In this respect, it should be interesting also to investigate whether VIP could regulate the release of adenosine or its conversion into inosine by the enzyme adenosine deaminase, which is now in progress in our laboratory.

In a recent report, Gressens and collaborators demonstrated that VIP increased after a 4-h incubation embryonic volume and somite number in whole postimplantation embryo cultures. In these experiments, a VIP antagonist, which is a hybrid peptide that contains a portion of VIP and a portion of neurotensin, completely suppressed VIP-stimulated mitosis in the CNS but decreased only partially the same in non-neuronal tissues. The data also support the fact that VIP action in these embryos is mediated through multiple VIP

receptor subsets that exhibit tissue-specific responses, a point that will be discussed later in the present review (Gressens et al., 1993). This report allows a caution concerning VIP antagonists utilized by various investigators. Some of these molecules are VIP derivatives and can be VIP fragments like the 10–28 sequence of the peptide presented elsewhere in this review; others are GRF derivatives (Waelbroek et al., 1985) or again hybrid molecules like the one utilized by the group of Gressens (Gozes et al., 1991; Gressens et al., 1993). One general remark concerning some of these molecules is that they need to be used in a narrow window of concentration where they do not possess agonist properties, but sufficient to efficiently compete with VIP at the level of the receptors. In this respect they should be considered like partial agonists rather than like authentic antagonists, according to the strict definition of this term.

Novel Data on VIP Receptors and VIP-Induced Signaling Pathways

A recent advancement in the knowledge of high-affinity VIP receptors has been brought by the demonstration that these binding sites are polyvalent and bind with similar affinity VIP and the structurally related polypeptide PACAP. Through these receptors, VIP and PACAP trigger with equivalent potencies their biological responses in target cells. However, specific PACAP receptors that do not interact with VIP have also been identified. For these reasons, it is now commonly accepted to refer to the specific PACAP receptors as PACAP type I and to the polyvalent VIP/PACAP receptors as PACAP type II (Shivers et al., 1991; Arimura, 1992). Cloning and expression of cDNAs sharing important sequence homologies and encoding distinct PACAP type I and PACAP type II binding sites substantiate this statement. These components belong to a distinct family of seven transmembrane spanning-domain G-protein coupled receptors, including the secretin, glucagon, GRF, calcitonin, and parathyroid hormone (PTH) receptors (Ishihara et al., 1992; Segre and Goldring, 1993). The data also support the notion of functional, pharmacological, and biochemical diversity of these binding sites proposed years ago (Laburthe and Couvineau, 1988; Luis et al., 1988; Meunier et al., 1992), since variants of these molecules are differentially expressed as a function of the tissue and species considered. However, in many tissues and some cell types, both PACAP type I and PACAP type II receptors appear to be coexpressed, although one subtype may be predominant (Shivers et al., 1991).

Hence, the polyvalent PACAP type II receptor corresponds to the "classical" high-affinity VIP receptor described years before the discovery of PACAP. Today, two distinct polyvalent VIP receptors are distinguished in a molecular point of view, in the light of the recent cloning of the corresponding cDNAs. The first form, named VIP_1, resembles the secretin receptor and is expressed in intestine, lung, and liver, as well as various brain regions, such as hypothalamus and cortex. The second, referred as VIP_2, is more related to the GRF binding site and has a distinct distribution in the CNS, with high expression in hippocampus, thalamus, and suprachiasmatic nucleus (Ishihara et al., 1992; Lutz et al., 1993). It is worth noting that these regions have been demonstrated to express a GTP-insensitive subtype of VIP receptor. Both GTP-sensitive and GTP-insensitive VIP receptors coexist in the mouse embryo, with highest expression in the CNS. It has been proposed that the growth-promoting actions of VIP in the mouse embryo CNS may be mediated by the GTP-insensitive sites (Hill et al., 1992; Gressens et al., 1993). In the rat brain, 90% of the earliest VIP receptors detected (at E. 16 and E. 18) in the embryo were GTP-insensitive and mostly localized in sites of glial formation (particularly the roof and floor plates). This indicates that VIP (or a related peptide, as discussed previously in this work) may act early in the brain ontogeny at GTP-insensitive binding sites, to regulate the multiplication and fasciculation of several glial populations in the CNS (Hill et al., 1994).

On the basis of their high affinity for the VIP analog helodermin, a third subtype of so-called "helodermin-preferring VIP receptors" is also recognized by some investigators in lung carcinomas and cells of the immune lineage (as reviewed in Lutz et al., 1993).

What Happens Inside the Cells After VIP Binds to Its Receptor?

The answers to this question are together promising and disappointing since important novel discoveries allow us to unveil some patches of the intracellular pathways that VIP may trigger; however, much needs to be done to understand the metabolic cascades that differentially govern, for instance, the VIP-induced inhibition or stimulation of cell proliferation. The most obvious cellular action of VIP is a transient increased cAMP production that triggers a metabolic cascade initiated by the activation of cAMP-dependent protein kinase and phosphodiesterase activities (Marchis-Mouren et al., 1988). This process is induced by concentrations of VIP lower than the nanomolar K_d values for the VIP receptors expressed in the corresponding tissues. However, although some investigators identified specific targets phosphorylated in response to VIP (Girault et al., 1988), these have not yet been clearly characterized, which leaves the subsequent steps of the cAMP initiated pathways highly speculative.

Stimulation by VIP of adrenal medulla was shown to increase the generation of inositol 1,4,5 triphosphate (IP3), to mobilize intracellular Ca^{2+} and to induce catecholamine secretion (Chick et al., 1988; Malhotra et al., 1988), which is to be brought together with the secretagog properties of VIP, and could explain how the peptide may facilitate vesicular traffic and exocytosis (Calderano et al., 1993). Similarly, production of inositol phosphates in response to VIP was observed in the superior cervical ganglion (Audigier et al., 1988), whereas a VIP-induced rise in intracellular Ca^{2+} has been reported in prolactin-producing rat anterior pituitary cells and in cultured rat hippocampal neurons (Tatsuno et al., 1992). It is worth noting that all these actions on the breakdown of phosphoinositides and Ca^{2+} rise were observed for relatively high concentrations of VIP, with maximal effects reached for micromolar peptide concentrations. However, it was recently reported that subnanomolar concentrations of VIP acting in synergism with α-adrenergic agonists produced large increases in intracellular calcium in some type I astrocytes from rat cerebral cortex. This synergism might mediate the VIP-induced mitogenic effect and/or the release of neurotrophic factors in astroglia (Fatatis et al., 1994). It has also been reported that internalized VIP could directly bind to calmodulin (Paul and Ebadi, 1993), but further data brought, for instance, by the utilization of calmodulin or Ca^{2+} channel inhibitors, are necessary to check the possible involvement of this process in the VIP-induced cellular and physiological responses.

It has been demonstrated that VIP activates nuclear protein kinase C in purified nuclei of rat splenocytes (Zorn and Russel, 1990). The authors proposed that this pathway may be triggered by the binding of internalized VIP to nuclear receptors, previously identified by other investigators in the human colonic adenocarcinoma cell line HT29 (Omary and Kagnoff, 1987). These statements of potential interest to understand cAMP-independent responses induced by VIP have not been substantiated by further characterization of the nuclear VIP receptors. A recent report describes that subnanomolar concentrations of VIP that did not produce an increase in intracellular cAMP levels, induced nuclear translocation of protein kinase C, particularly of the alpha subtype, in neonatal rat cortical astrocytes (Olah et al., 1994) These observations support the statement that some signal transduction pathways elicited by VIP are independent of cAMP. Such signaling processes may play a role in mediating glial response to VIP, and subsequently in regulating the neuron–glia interactions governing neurodevelopment.

Another avenue of investigation concerns the inhibition by VIP of phospholipase A_2 activity from porcine pancreas and cobra venom. The activity of this enzyme, which generates arachidonic acid, the precursor of all eicosanoids, is increased in inflammatory injuries. The potential anti-inflammatory properties of VIP may contribute to the protective effects of the peptide observed in the lung and other tissues, for instance, prevention by the peptide of neuronal cell killing by the gp120 envelope protein of the HIV virus (Brenneman et al., 1988; Said, 1990; Trotz and Said, 1993; Wei and Thomas, 1993).

Concerning the protective effects of VIP, the demonstration that VIP induces the production of interferon α/β synthesis in the human colonic adenocarcinoma cell line HT29 and in glial tissue but not in neurons deserves to be cited here (Chelbi-Alix et al., 1991, 1994); this process may be related to antiviral-inducing properties of VIP but also to other actions, including mitogenic regulation of the interferons released in response to VIP by target cells.

Induction of smooth muscle relaxation was among the very first reported actions of VIP and still remains the subject of exciting breakthroughs in the knowledge of the modalities of VIP action. As a matter of fact, recent evidence based on the use of nitric oxide synthase (NO) inhibitors demonstrates the existence of an interplay between VIP and NO in the regulation of smooth muscle relaxation, since NO produced in nerve terminals facilitates VIP release and VIP in turn stimulates further NO production in target muscle cells (Grider et al., 1992). Physiological involvement of NO in the VIP-induced smooth muscle relaxation has been demonstrated in tracheally superfused guinea pig lungs (Lilly et al., 1993). In rabbit gastric muscle cells, VIP increases both cAMP and cGMP, which results in the activation of cyclic nucleotide-dependent protein kinases and in muscle relaxation; the increase in cGMP most likely results from stimulation of nitric oxide synthase and activation of soluble guanylate-cyclase by nitric oxide. In this process, activation of the soluble NO synthase appears to be mediated through a VIP-induced G protein-coupled rise of intracellular Ca^{2+} levels and subsequent activation of the Ca^{2+}/calmodulin complex, since this process is abolished by Ca^{2+} channel and calmodulin antagonists. Furthermore, the data demonstrate that two subsets of VIP receptors are coexpressed in this tissue, one that is referred to as VIP-specific that mediates the cascade leading to activation of NO synthase and that does not recognize PHI, and the second that is positively coupled to adenylate-cyclase and also interacts with PHI (Murthy et al., 1993). Interestingly, the VIP-specific receptors do not appear to be desensitized, suggesting that they may mediate long-lasting effects of VIP. It is worth noting that constitutive Ca^{2+}/calmodulin-dependent NO synthases are present in neurons and glial cells (Bredt and Snyder, 1992) and play important neurobiological roles, including long-term potentiation of neurotransmitter impact, particularly of glutamate action through NMDA receptors (Bon et al., 1992). These novel and important clues will certainly lead to thrilling developments concerning the studies of cAMP independent mechanisms induced by VIP in target cells.

Considering these data, we undertook a comparative pharmacological identification of the [^{125}I]-VIP binding sites expressed in neuroblastoma SH-IN subclone and in the colonic adenocarcinoma cell line HT29. The idea was to check if the opposite actions of VIP in these different tissues could be correlated to differential expression of VIP receptor subsets in these cell lines, leading to the observation of quite different pharmacological profiles. The data presented in Fig. 2A,B demonstrate that the profiles of these sites are very similar in these human cell lines, which in this case rules out the working hypothesis. However, in both tissues, PHM (the human PHI analog) never totally displaced [^{125}I]-VIP binding, even at the highest concentrations utilized, demonstrating that specific VIP-receptors that do not bind PHM may be expressed in the cell lines considered.

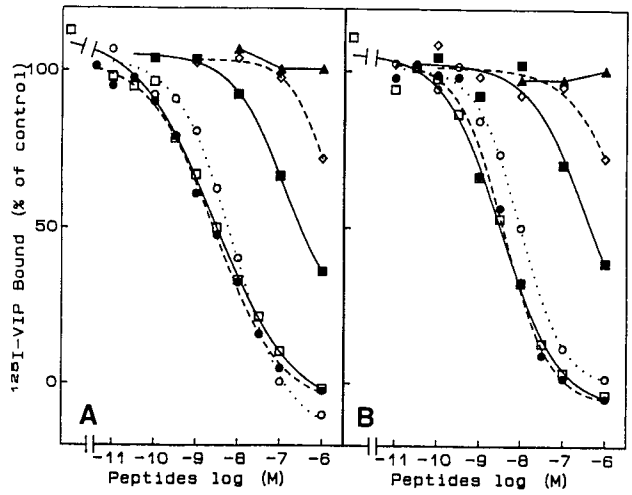

Fig. 2. Competitive inhibition of specific [^{125}I]-VIP binding by unlabeled VIP and structurally related peptides in **(A)** HT 29, human colonic adenocarcinoma cells, and **(B)** SH-IN, human clonal neuroblastoma cells. Cells (10^5 for HT 29 and 5×10^5 for SH-IN) were seeded in 24-well culture plates 3 d before binding in DMEM supplemented with 10% FCS and antibiotics. Culture medium of SH-IN was changed 3 h before binding, since these cells release immunoreactive VIP. Binding was performed in the culture wells, in DMEM containing 15 m*M* HEPES (pH 7.4), 1% BSA, 0.1% bacitracin, and 150 µ*M* phenyl methyl sulfoxide *(binding medium)*. Cells were incubated (after a 30-mn pre-equilibration at 37°C for SH-IN) at 4°C for 3 h in presence of 30 p*M* [^{125}I]-VIP and increasing concentrations of VIP (□ ——), PACAP 27 (● ---), PACAP 38 (○ ...), PHM (■ ——), secretin (◇ ---), and glucagon (▲——). Curves were fit by nonlinear regression analysis using the program GRAPHPAD. Each point represents the mean (n = 6) ± SEM (<3%).

Summary: Conclusion and Perspectives

The wide distribution of VIP and the large spectrum of biological activity that takes place relatively early in ontogeny allow us to speculate that the peptide may possess developmental functions, particularly in neural tissue. The regulation of the release of VIP as a function of neuronal electrical activity and the control of the VIP precursor gene expression by steroids, retinoic acid, or by cAMP and calcium-dependent mechanisms, appear like many principles able to govern the fine tuning of the VIP impact in neurodevelopment. In addition to its commonly recognized status as a neuromodulator of virtually all vital functions, including neurobiological, VIP plays a role in the control of cell growth and differentiation and of neuronal survival. This property is shared by other neuropeptides, including ACTH, the calcitonin gene related polypeptide (CGRP), vasopressin, substance P, and neurokinin A (as reviewed by Gozes and Brenneman, 1993). Important questions are whether VIP possesses intrinsic mitogenic activity or whether it regulates the action or the release of growth and trophic factors. The data presented here indicate that the actions of VIP in the control of cell proliferation and differentiation are more readily observed in cells cultured in the presence of serum as a source of growth factors, in Con A stimulated lymphocytes, or again in preparations of dissociated spinal cord containing mixed neuroblasts and glial cells. This general remark immediately suggests that VIP action is most likely significant when mitogenic or trophic factors, eventually released by nontarget cells, are simultaneously present in the extracellular medium. One exception is its direct stimulatory action on the proliferation and IgA production in B-lymphocytes that can take place in the absence of serum or other exogenous mitogenic substances, which does not rule out the possible involvement in this process of endogenous lymphokines released in response to VIP. In these cells, but also in T-lymphocytes, VIP did not significantly stimulate cAMP production, suggesting that some effects of the peptide may be mediated by mechanisms that are independent of the cyclic nucleotide.

Another interesting consideration is that VIP impact on cell growth can be stimulatory or inhibitory as a function of the model considered. These opposite effects of VIP are observed in neuronal or neuroblastoma cell types in which the peptide may often act in an autocrine manner. Inhibition, but not stimulation, by VIP of cell proliferation is frequently

associated with the development of differentiation in target cells, suggesting possible crosstalks between the corresponding signaling pathways. Differentiation usually takes place after several days of treatment in the presence of VIP and can be mimicked by compounds, like forskolin, that potently stimulate adenylate-cyclase activity, or by cAMP analogs, like dibutyril cAMP. However, it is well recognized that elevation of intracellular cAMP level in response to VIP is very transient; furthermore, the peptide present in the extracellular medium is rapidly internalized and degraded by receptor-mediated endocytosis and other processs, making a long-lasting impact of extracellular VIP unlikely. Hence, the long-term action of VIP on cell growth and differentiation probably results in mechanisms whose duration may be related to subsequent regulation of the expression and activity of key components involved in the interrelated controls of cell cycle and differentiation. These components may be as diverse as transcriptional regulators, like the *jun/fos* complex, *c-myc*, or the p53 protein, or again distinct phosphorylation cascades involving, for instance, small G proteins, Raf_1 and the MAP-kinases (Berthon et al., 1992). An alternative is that VIP could regulate the liberation of trophic and/or mitogen factors having long-term autocrine and/or paracrine activities, which could be polypeptidic or not, like nitric oxide, prostaglandins, or extracellular adenosine. These compounds, which may be liberated either by the same cells that react to them or by neighboring ones, may relay the transient initial action of VIP. This possibility is most likely one important principle governing neurodevelopmental actions of VIP and its effects on neuronal survival, since the demonstration that the peptide induces the release of neurotrophic factors from glial tissue is now well established.

To conclude, we propose that VIP can be considered like a neuromodulator, directly regulating the impact of growth and differentiation factors in neural tissue, eventually in an autocrine fashion. Indirectly VIP may control neuronal behavior through its action on the release of glial neurotrophic and growth factors. The impact of VIP appears early in ontogeny but peaking expression of the VIP precursor mRNA is observed likewise in the newborn, during critical periods of brain maturation. The intracellular mechanisms that mediate these actions of VIP may involve different transduction cascades triggered by different subsets of VIP binding sites that may coexist in the same tissue:

1. A subtype interacting with both VIP and PHI, coupled via a G protein to transient activation of adenylate-cyclase as a consequence of their rapid desensitization;
2. A subtype that is not coupled to adenylate-cyclase but to a G-protein and Ca^{2+}/calmodulin-dependent activation of nitric oxide and cGMP production; these receptors are poorly desensitized and may ensure long-lasting actions of VIP;
3. Very high affinity receptors that are not coupled to G proteins and that appear to mediate the mitogenic actions of low concentrations of VIP in some brain areas.

These receptors appear early in brain ontogeny, and may be involved in gliogenesis and release of neurotrophic molecules.

Further characterization of these different receptor subtypes and exploration of the transduction cascades triggered by them should allow the pathways governing the opposite effects of VIP in the regulation of cell growth and differentiation to be better understood. Concurrently, studies should be developed toward the nature of the growth, differentiation, and trophic factors whose release is regulated by VIP and that exert key developmental actions.

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