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Review

Unconventional binding sites and receptors for VIP and related peptides PACAP and PHI/PHM: An update

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ABSTRACT

The 28-amino-acid neuropeptide VIP and related peptides PACAP and PHI/PHM modulate virtually all of the vital functions in the body. These peptides are also commonly recognized as major regulators of cell growth and differentiation. Through their trophic and cytoprotective functions, they appear to play major roles in embryonic development, neurogenesis and the progression of a number of cancer types. These peptides bind to three well-characterized subtypes of G-protein coupled receptors: VPAC1 and VPAC2 share a common high affinity in the nanomolar range for VIP and PACAP; a third receptor type, PAC1, has been characterized for its high affinity for PACAP but its low affinity for VIP. Complex effects and pharmacological behaviors of these peptides suggest that multiple subtypes of binding sites may cooperate to mediate their function in target cells and tissues. In this complex response, some of these binding sites correspond to the definition of the conventional receptors cited above, while others display unexpected pharmacological and functional properties. Here we present potential clues that may lead investigators to further characterize the molecular nature and functions of these atypical binding species.

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1. Introduction

The 28-amino-acid neuropeptide VIP (vasoactive intestinal peptide), isolated from the small intestine, derives from a propeptide which gives also rise to a VIP analogue, the 27-amino-acid PHI/M (peptide histidine isoleucine, or its human counterpart peptide histidine methionine). These molecules are members of the secretin-like peptide family which also includes glucagon and Growth hormone releasing factor (GRF). Along with PACAP (pituitary adenylate-cyclase activating polypeptide), the structurally similar 27- or 38-amino-acid long peptide, VIP displays a very large spectrum of biological activities. These peptides modulate virtually all of the vital functions in the body through typical physiological effects: visceral smooth muscle relaxation, stimulation of exocrine and endocrine secretion, and regulation of major metabolisms, such as glycogenolysis and lipolysis. They are the main neurotransmitters in the gut and both play a neuromodulatory role in the central and peripheral nervous systems, in neuronal cells as well as in glial cells. One prominent signaling pathway of VIP/PACAP is the stimulation of adenylate-cyclase activity. However, other transduction cascades including phospholipases C or D, mitogen-activated protein kinase (MAPK) or NO synthase associated pathways have also been shown to be controlled by these neuropeptides [3,54,59]. These peptides are also commonly recognized as major regulators of cell growth and differentiation, with trophic and cytoprotective functions, playing major roles in embryonic development, neurogenesis and the progression of a number of cancer types [13,16,36,37,39,40,52,61]. The multiple and potent regulatory properties of VIP and related peptides led to the proposal that these molecules or synthetic agonists or antagonists may lead to interesting clinical applications in various types of diseases such as cancer, diabetes, impotence, inflammation, asthma, cystic fibrosis and neurodegenerative disorders. Some VIP and related peptide derivatives have indeed been developed, but their relative specificity for the different VIP or PACAP receptor subtypes in clinical treatments could lead to unwanted side effects, which is a limitation for further therapeutic developments [4,20]. Hence, a better knowledge of these neuropeptide receptors and the associated intracellular transduction pathways is needed in order to develop novel peptidic or non peptidic analogues with higher specificity and efficiency.

VIP and PACAP share a wide spectrum of biological activity and common receptors belonging to the so-called class II of G-protein coupled receptors (GPCR). Three VIP or PACAP receptor types deriving from independent genes are officially recognized and named VPAC1, VPAC2 and PAC1 by IUPHAR (International Union of Pharmacology) [24]. In this review, they will be referred to as the ''conventional VIP and PACAP receptors.'' Two of these receptors, VPAC1 and VPAC2, share a common high affinity in the nanomolar range for VIP and PACAP. A third receptor type, PAC1, has been characterized for its high affinity for PACAP but low affinity for VIP [24,28,59]. The aim of the present review is to briefly summarize and update a significant array of experimental data supporting the concept that multiple subtypes of binding sites may cooperate to mediate VIP and related peptides' effects in target cells and tissues. In this complex response, some of these binding sites correspond to the definition of the conventional receptors cited above, while others display unexpected pharmacological and functional properties. These peculiar binding sites correspond to high-affinity VIP-selective or PHI/PHM selective subtypes, or to GTP-insensitive VIP binding components. This raises the question of the molecular nature of the ''unconventional'' receptors corresponding to these binding sites. Here we present potential clues that may lead investigators to further characterize the molecular nature and the function of these atypical binding species.

2. Main properties and multiple isoforms of conventional receptors for VIP and related peptides

2.1. *The VPAC1 and VPAC2 receptors*

Several excellent recent reviews have been dedicated to these receptors [28,59], and we will briefly summarize some of their main properties. These receptors derive from two independent genes and share a common high affinity for VIP and PACAP. Along with their natural ligands, they are widely distributed in the body, in peripheral tissues and in the nervous, neuroendocrine and immune systems. In the brain, however, VPAC1 and VPAC2 are expressed in distinct areas, VPAC2 distribution being more restricted [5,28,55].

The pharmacological profiles of the human VPAC receptors expressed in CHO cells are as follows (average K_d values are indicated between parenthesis):

VPAC1 receptor:

$$\begin{aligned}\text{VIP} = \text{PACAP27}\ (1\,\text{nM}) &> \text{PACAP38}\ (6.8\,\text{nM}) \\ &> \text{Helodermin}\ (46\,\text{nM}) > \text{GRF}\ (0.6\,\mu\text{M}) > \text{PHM}\ (2\,\mu\text{M}) \\ &> \text{secretin}\ (10\,\mu\text{M})\end{aligned}$$

VPAC2 receptor:

$$\text{PACAP38 (0.8 nM)} = \text{VIP (0.9 nM)} > \text{helodermin (2.5 nM)} = \text{PACAP27 (3 nM)}$$

The VIP analogue Helodermin, isolated from lizard venom, discriminates between the two types of VPAC receptors and is considered a relatively selective ligand of the VPAC2 receptor. The VPAC1 receptor and particularly its N-terminal ectodomain has been extensively studied using different approaches of site-directed mutagenesis, photoaffinity probes, nuclear magnetic resonance, molecular modeling and chimeric receptor constructs [14,28,29,57]. These studies helped to determine crucial residues and domains involved in ligand binding and internalization, receptor activation, coupling and desensitization, as well as receptor specificity toward different agonists and antagonists. Much more limited data have been reported on the VPAC2 receptor. Recently, inactivating mutations were generated by site-directed mutagenesis, which allowed for determination of key residues involved in phosphorylation and internalization of the human VPAC2 receptor [30].

Novel data concerning the knowledge of these receptors come from studies on truncated five-transmembrane (5TM) isoforms of both human VPAC1 and human VPAC2. The corresponding alternatively spliced variant mRNAs result from the skipping of exon 10/11 of the genes (composed of 13 exons), spanning the third intracellular loop, the fourth extracellular loop, and the transmembrane regions 6 and 7, producing in-frame 5TM receptors predicted to lack a G-protein-binding motif. The 5TM VPAC1 was less frequently expressed than the 7TM isoform, as analyzed in different cancer cell lines, using a specific nested RT-PCR technique. Expression of the 5TM isoform was observed in a few cancer types deriving from epithelium, colon or hematopoietic tissue and in normal peripheral blood mononuclear cells. Agonist stimulation of the 5TM VPAC1 receptor expressed in CHO cells did not result in activation of adenylate-cyclase, while induction of tyrosine phosphorylation was observed in transfected cells in response to high concentrations of VIP or secretin [8].

2.2. *The PAC1 receptor*

The PAC1 receptor was initially called ''PACAP-preferring'' receptor because of its high selectivity for PACAP. In fact, in the initial studies of expression of the human PAC1 in transfected CHO cells, the receptor displayed a high affinity for PACAP27 or 38 in the nanomolar range, and a 1000-fold lower affinity for VIP of about 1 μM [3,59]. Later, numerous isoforms of the PAC1 receptor, corresponding to 17 known splice variants of the same gene, were identified [33]. The human PAC1 gene is composed of 18 exons [9], ten being constitutively expressed (exons 2, 3, 7–13 and 18) while seven (exons 4–6 and 14–17) are regulated [33]. A complex process of differential splicing generates the numerous receptor isoforms that display distinct pharmacological profiles and coupling to intracellular effectors. Most of them behave as specific PACAP receptors, but some, like the newly discovered human delta 5–6 splice variant (lacking the domains corresponding to exons 5 and 6), appears to share a similar nanomolar affinity and is efficiently activated by both VIP and PACAP [33]. Considering as a reference the so-called ''null'' PAC1 receptor, the other isoforms are characterized by the absence (''short'' form) or the presence of 2 cassettes of 28 (''hip'' or ''hop1'' form) or 27 (''hop2'') amino acids in the third intracellular loop. A species lacking 21 amino acids in the N-terminal domain was called ''very short.'' These variations can then be combined with those resulting from deletions of exon 5 or 6, or both, increasing the number of isoforms deriving from the PAC1 gene. Hence, one has to consider that the PAC1 gene can give rise to ''PACAP-preferring'' receptors, but also to variants like the delta 5–6 isoforms that share with the VPAC receptors a similarly high affinity for VIP and PACAP [33]. Moreover, a recent report suggests that the rat hop2 PAC1 isoform also acts as a high affinity receptor for both VIP and PACAP [46]. This PAC1 variant appears to mediate the cytoprotective effects attributed to VIP [46].

3. Complex effects and pharmacological behaviors of VIP and related peptides suggest a multiplicity of binding sites for these neuropeptides

3.1. *VIP-selective or ''preferring'' binding sites*

The conventional VIP receptor subtypes VPAC1 and VPAC2 that display a high-affinity for both VIP and PACAP probably mediate most of the common effects of these peptides in target cells and tissues. However, it has been demonstrated that some biological effects of VIP are not reproduced by PACAP 27 or 38. An example is represented by studies from the group of P. Gressens on VIP-induced neuroprotection in postnatal day 5 mouse brain with white matter lesions mimicking human periventricular leukomalacia. In this model, intra-cerebral co-injected VIP protects against white matter lesions induced by the glutamate agonist ibotenate. This neuroprotection is independent from cAMP and is mediated by protein kinase C. VIP effects were mimicked with a similar potency by VPAC2 agonists and PHI but not by VPAC1 agonists, PACAP 27 or PACAP 38. VIP's neuroprotective effects were lost in mice lacking the VPAC2 receptor. In situ hybridization confirmed the presence of VPAC2 mRNA in the postnatal day 5 white matter [50]. When analyzed between embryonic life and adulthood, VIP specific binding sites density peaked at postnatal day 5. These data suggest that, in this model, VIP-induced neuroprotection is mediated by VPAC2 receptors. However, the pharmacology of this VPAC2 receptor appears quite unconventional, since in this model: (i) PACAP did not mimic VIP effects, (ii) PHI acted with a comparable potency and (iii) PACAP 27 modestly inhibited the VIP specific binding, whereas for PHI or VIP inhibition was complete [50,51].

Studies from our own group also observed VIP-specific binding sites in the C6 glioblastoma cell line [17]. Effects of VIP and related peptides on cell proliferation were assayed in the C6 rat glioblastoma cell lines. 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 those 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 [17].

Recently, the cloning of the full-length cDNA of a VIP-specific receptor from guinea pig tenia coli smooth muscle has been reported [63]. The cDNA sequence of this receptor encodes a 437-amino acid protein that possesses 87% similarity to mouse and rat VPAC2 receptors and differs from the guinea pig gastric smooth muscle VPAC2 receptor by only two amino-acid residues, F(40)F(41) in lieu of L(40)L(41). This receptor expressed in transfected COS-1 interacted only with VIP with high affinity (IC50 = 1.4 nM) and efficiently stimulated cAMP formation with high potency (EC50 = 1 nM). Hence this VIP-specific receptor cloned from guinea pig teniae coli smooth muscle could be distinct from VPAC1 and VPAC2 receptors [63].

3.2. *ANP-C receptors allow crosstalk between VIP related peptides and Atrial natriuretic peptide*

Atrial natriuretic peptide (ANP) and the closely related peptides BNP and CNP are highly conserved cardiovascular hormones. They activate two subtypes of single transmembrane-spanning receptors, NPR-A and NPR-B, triggering receptor-intrinsic guanydylate-cyclase activity. Interactions between PAC1 and VPAC2 receptors and the NPR-B subtype have been reported in cultured rat corpus cavernosum smooth muscle cells. Prior exposure of these cells to 10 nM PACAP resulted in a marked down-regulation of NPR-B, suggesting a cross-talk of the PACAP/VIP receptors with the B subtype of ANP receptor, a process which may have implications for the therapy of erectile dysfunction [22]. A third receptor for these peptides is represented by the ''truncated'' type-C natriuretic peptide receptor (NPR-C), which has long been called a clearance receptor because it lacks the intracellular guanydylate-cyclase domain, though data suggest it might negatively couple to adenylate-cyclase via G(i). Independent studies suggested that NPR-C might be a polyvalent receptor for natriuretic as well as VIP-related peptides [6,42]. The molecular cloning and characterization of the *Xenopus laevis* type-C natriuretic peptide receptor (XNPR-C) reveals that this component displays a high similarity to fish and mammalian NPR-C, particularly the presence of a short intracellular C-terminus [32]. Injection of XNPR-C mRNA into *Xenopus* oocytes resulted in expression of high affinity ^{125}I-ANP binding sites that were competitively and completely displaced by natriuretic analogues but also by the unrelated neuropeptide VIP with very similar IC50 values in the nanomolar range. Measurement of cAMP levels in mRNA-injected oocytes revealed that XNPR-C is negatively coupled to adenylate-cyclase in a pertussis toxin-sensitive manner. When XNPR-C was co-expressed with PAC1 receptors, VIP and natriuretic peptides counteracted the cAMP induction by PACAP. These results suggest that VIP and natriuretic peptides can potentially modulate the action of PACAP in cells where XNPR-C and PAC1 receptors are co-expressed [32].

Another level of complexity in the action of VIP and related peptides comes from the expression in a number of tissues of high-affinity PHI/PHM binding sites which also interact with VIP, at least for one part of them (see below). Data concerning the knowledge of these PHI/PHM binding sites, which have been characterized using radioiodinated derivatives of PHI or PHM, are presented in the next section.

4. The enigma of the high-affinity PHI/PHM binding sites

The pharmacological profiles of VPAC1 and VPAC2 indicate that these receptors interact with PHI/PHM with a modest affinity, so that these peptides were considered low affinity agonists for the conventional VIP receptors. However, expression of high affinity PHI or PHM binding sites in different tissues has been reported by several independent groups, using ^{125}I-PHI or ^{125}I-PHM [31,44,45,50]. One of the earliest studies on PHI/PHM binding sites was conducted on rat liver membranes, using ^{125}I-PHI in the presence of a non-solubilizing concentration (1 mM CHAPS or 0.01% Tween-20) of detergent, in order to reduce adsorptive loss of PHI to acceptable levels and permit measurement of PHI binding to receptors [44]. Under these conditions, unlabeled PHI was 9.7-fold more potent than VIP, and 357-fold more potent than secretin in displacing ^{125}I-PHI binding. Scatchard analysis suggested the presence of two classes of PHI receptors, with K_d 27 and 512 pM. Comparison of the data from ^{125}I-PHI and ^{125}I-VIP binding studies suggested that one class of receptors was ''PHI-preferring'', and the other equally reactive with PHI and VIP. These studies on high affinity PHI/PHM binding sites raised the question of the nature of the corresponding receptors. It has been demonstrated that only some of the high affinity VIP binding sites also interact with PHI/PHM with IC50 values similar to VIP itself. One example is represented by studies on VIP-induced signal transduction events involved in rabbit gastric smooth muscle relaxation. In this model, VIP increases both cAMP and cGMP. The VIP-induced increase in cGMP most likely resulted from nitric oxide (NO) synthase activation and consequently of NO-dependent soluble guanylate-cyclase. In this process, activation of NO-synthase appeared 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. The data also demonstrated that two subsets of VIP receptors are coexpressed in this tissue: one, referred to as ''VIP-specific,'' mediated the cascade leading to activation of NO synthase and did not recognize PHI, and another that was positively coupled to adenylate-cyclase and interacted with both VIP and PHI [41]. VIP-specific binding sites sharing a similarly high affinity for VIP and PHI have been observed in post-natal rat brain. These sites, which mediate efficient and sensitive neuroprotective effects of VIP and PHI but not of PACAP, may correspond to VPAC2 receptors expressed in this tissue [50,51].

Studies from our own group on the mouse neuroblastoma cell line Neuro2a demonstrated that PHI and VIP inhibit proliferation at concentrations as low as 0.1 pM and 0.9 nM, respectively. Peptide actions were studied further by measuring cAMP and ERK1/2 MAP kinase activity and by assessing ^{3}H-thymidine incorporation in conjunction with a panel of signal transduction pathway inhibitors. The data obtained indicated that the PHI-inhibitory activity was mediated by corresponding changes in activity of the MAP kinase pathway, independent of protein kinase A (PKA) or protein kinase C (PKC). In contrast, the inhibitory action of VIP was specifically blocked by antagonists of PKA. Northern blot analysis revealed gene expression for only the ''PACAP-preferring'' (PAC1) receptor. However, binding experiments using ^{125}I-labeled PACAP27, PHI, or VIP, demonstrated the presence of ''PACAP-preferring'' sites, bivalent VIP/PACAP sites, and high affinity PHI-binding sites that did not interact with VIP and could thus be called ''PHI-preferring''. These studies demonstrate that the potent regulatory effects of PHI and VIP on neuroblastoma cell proliferation appears to be mediated by multiple subsets of receptors which differentially couple to MAP kinase and PKA signaling pathways [31].

Another interesting advancement in the knowledge of PHI/PHM binding sites was the demonstration that at least some of them correspond to GTP-insensitive high affinity binding sites. In other words, the peptides PHI and PHM appear to be interesting pharmacological tools to distinguish the GTP-insensitive VIP binding sites. Since such observations open new interrogations concerning the nature of these GTP-insensitive binding sites, they will be summarized in the next section, after a short review of the data that led to their discovery.

5. GTP insensitive VIP binding sites: possible clues

5.1. *Origins of the concept of GTP-insensitive VIP binding sites*

The scheme of the functional cycle of G proteins and GPCR activity indicates that interaction of an agonist with its cognate GPCR promotes the release of GDP from the alpha subunit of the heterotrimeric G protein, resulting in the formation of GTP-bound G alpha. GTP-G alpha and G beta/gamma dissociate and are able to modulate effector functions. In this configuration, the GPCR is left in a low affinity state until it again associates with G proteins, after GTP hydrolysis by G alpha and associated proteins, such as RGS (regulator for G-protein signaling). Experimentally, this phenomenon corresponds to a strong inhibition of receptor/ligand interaction in the presence of an excess of GTP or a non hydrolysable guanosine 5′-triphosphate analogue, such as guanydylate-imidodiphosphate (GppNHp) [62].

Utilization of GppNHp allowed the initial observations of GTP-insensitive VIP binding sites, reported long ago in the rat liver [1,49]. Later, these sites were purported to play important functions in the early stages of embryonic brain development. Studies from Hill's group, using in vitro autoradiography of ^{125}I-VIP, led to the demonstration that two subtypes (or different functional states of a single subtype) of VIP binding sites are represented in the rat brain [26]. In most brain regions, GppHNp reduced VIP binding between 40 and 60%. However, in some areas, such as the supraoptic nucleus, locus coeruleus, interpeduncular nucleus, facial nucleus, olfactory tubercle and periventricular hypothalamic nucleus, 80% or more of vasoactive intestinal peptide binding was inhibited. In other brain regions, including the medial geniculate, olfactory bulbs, and ventral thalamic nuclei, GppNHp had little effect on vasoactive intestinal peptide binding. In the liver, lung and intestine it also partly inhibited VIP binding. Electrophoretic analysis of ^{125}I-VIP, covalently cross-linked to its receptors in brain membranes, revealed a pair of bands between 44 and 52 kDa, a component at 64 kDa and another at 92 kDa. All were displaceable with unlabeled VIP. However, GppHNp displaced only the 64 kDa band, suggesting that the GTP-sensitive vasoactive intestinal peptide receptor present in brain sections corresponds to a roughly 61 kDa component after subtraction of VIP molecular weight; the other components, particularly the 44–52 kDa doublet, behaved like GTP insensitive species. Hence, based on their differential sensitivity to GppNHp, at least two VIP receptor binding components are represented in the brain, with distinct regional distribution, probably reflecting differential coupling to second messenger systems [25]. The pattern of expression of these receptor subtypes was demonstrated to evolve throughout rat CNS development from embryonic day 14 (E14) to adult. The changing patterns were the result of (1) the transient appearance of GTP-insensitive VIP receptors in several regions undergoing mitosis or glial fasciculation, and (2) the transient appearance of GTP-sensitive VIP receptors homogeneously distributed throughout the CNS during the first two postnatal weeks, the period of the brain growth spurt.

A spectacular action of VIP is represented by its trophic and mitogenic properties on embryonic neural tissues that were analyzed on whole postimplantation embryo cultures. After a 4-h incubation, VIP stimulated growth, increasing somite number, embryonic volume, DNA and protein content, and number of cells in S-phase. A VIP antagonist substantially inhibited these VIP-mediated increments in growth. The VIP antagonist completely suppressed VIP-stimulated mitosis in the CNS while decreasing the same in non-neuronal tissues by 38%. In vitro autoradiography revealed GTP-sensitive and GTP-insensitive VIP receptors which were differentially regulated in VIP antagonist-treated embryos. This study suggested that VIP acts as a growth factor on early postimplantation embryos through multiple VIP binding sites, including GTP-insensitive species that exhibited tissue-specific responses [21].

5.2. *GTP-insensitive VIP binding sites are discriminated by PHI*

Early reports on ^{125}I-VIP binding experiments in liver membranes proposed that the VIP binding sites were partially sensitive to GTP [1,49]. Studies from our group confirm that the VIP binding sites of chicken liver membranes consist mainly in bivalent VIP/PACAP receptors and that about 50% of the ^{125}I-VIP binding capacity is not affected by the GTP analogue GppNHp. Part of these bivalent receptors also appeared to represent PHI binding sites. In GppNHp-treated membranes,

the GTP-insensitive VIP binding sites displayed a 17-fold higher relative affinity than in control membranes for the VIP analogue PHI. Such data suggest that high-affinity PHI binding sites correspond to GTP-insensitive VIP receptors. Cross-linking of ^{125}I-VIP or ^{125}I-PHI in liver membranes revealed two components of 48 and 60 kDa. The radiolabeling of the 60 kDa component was strongly affected by increasing concentrations of GppNHp but was modestly abolished by an excess of PHI. Conversely, the radiolabeling of the 48 kDa molecular form was not affected by the GTP analogue but was efficiently abolished by increasing concentrations of PHI. Taken together, the data suggest that the GTP-insensitive VIP/PHI receptor in this tissue corresponds to a 48 kDa species distinct from a GTP-sensitive 60 kDa VIP binding component that poorly interacted with PHI [45].

We recently reported that proliferation of the human lymphoblastoma cell line H9 was differently stimulated by peptide histidine methionine (PHM) and vasoactive intestinal peptide (VIP). PHM induced a cyclic AMP (cAMP) accumulation, abolished by adenylate-cyclase (AC) inhibitors leading to a loss of proliferative effect. VIP mitogenic activity was Pertussis toxin (PTX) sensitive and AC inhibitor insensitive. Pharmacological experiments performed on H9 membranes with or without GppHNp indicated expression of both GTP-insensitive and -sensitive PHM/VIP high-affinity binding sites (HA). Of the three conventional VIP/PACAP receptors, only VPAC1 was found to be expressed in H9 cells. VIP(10-28), corresponding to the C-terminal part of the peptide, is known as a VPAC1 antagonist. This analogue interacted with all GTP-insensitive PHM binding sites and inhibited both the PHM and VIP mitogenic actions. These data suggest that VIP and PHM regulate H9 cell proliferation through distinct mechanisms and highlight the key role of GTP-insensitive binding sites in the control of cell proliferation [19].

5.3. *The PHI/PHV goldfish receptor*

Peptide histidine isoleucine (PHI), peptide histidine valine (PHV), and vasoactive intestinal polypeptide (VIP) are cosynthesized from the same precursor, PHV being an extended form of PHI. An high affinity PHI/PHV receptor was isolated and characterized in the goldfish. Functional expression of this PHI/PHV receptor in Chinese hamster ovary (CHO) cells revealed that it could be efficiently activated by human PHV (and to a lesser extent human PHI and helodermin) but not fish and mammalian PACAP and VIP. Of course, this receptor was also able to interact with goldfish PHI and its C-terminally extended form, PHV. Structurally as well as pharmacologically (due to its relatively high affinity for helodermin), this component resembles the VPAC2 receptor. Northern blot and RT-PCR/Southern blot analyses revealed that the PHI/VIP gene is expressed in the intestine, brain, and gall bladder, and the PHI/PHV receptor gene is primarily expressed in the pituitary and to a lesser extent in the intestine and gall bladder, suggesting that PHI/PHV may play a major role in the regulation of hypophyseal function. These studies demonstrate the existence of a receptor responding to the definition of ''PHI-preferring'' binding sites that could selectively mediate the effects of PHI or PHV, in addition to conventional VIP receptors [58]. However, no novel GPCR equivalent to the goldfish PHI/PHV receptor has so far been characterized in mammals.

5.4. *VPAC2 receptors expressed in transfected CHO cells generate GTP-insensitive high affinity PHM binding sites*

Until now, the molecular nature of the GTP insensitive binding sites has not been clearly identified. Do they correspond to unknown novel receptors or to a conformational state of the identified VIP receptors, due to their association with distinct components present in a specific membrane environment?

To answer this question, some insight from unpublished data from our group is presented here. We first tested the GTP sensitivity of the two known high-affinity bivalent human VIP and PACAP receptors, VPAC1 and VPAC2, in stably transfected CHO cells, using ^{125}I-PHM. Binding experiments were carried out on freshly prepared cell membranes according to conditions described previously [15,45]. Briefly, CHO cell membranes (10 μg) were incubated with ^{125}I-PHM and various concentrations of unlabeled PHM in the presence or absence of 20 μM GppNHp. In this system, PHM binding displayed a different pharmacological behavior towards the two VPAC receptors: ^{125}I-PHM binding capacity was mainly sensitive (80%) to GppNHp on the VPAC1 (not shown), while most of the radiolabeled peptide binding (75%) was GppNHp insensitive on the VPAC2 receptor (Fig. 1A). This suggests that in the context of the CHO cells, VPAC receptors could correspond to the GTP insensitive binding sites observed long ago by Hill et al. in the newborn rat brain [25]. This allows the hypothesis that the differential localization of GTP sensitive or insensitive binding sites may be due to a change in the state of these same receptors, rather than a differential distribution of receptor subtypes in the rat central nervous system. Interestingly, the distribution of the VAPC2 receptors in the CNS correlate quite well to that of the GTP-insensitive binding sites. The data also indicate that PHM was able to bind with a high affinity (in the nM range) and in a GTP sensitive manner to the VPAC1 receptor, while PHM binding on VPAC2 receptor was mainly insensitive to the nucleotide. This makes of VPAC2 an interesting candidate for the status of GTP-insensitive high affinity ''PHM-preferring'' receptor.

6. Modulation of VIP receptors pharmacology: role of accessory proteins

6.1. *The receptor activity-modifying proteins (RAMP) modulate VPAC1 receptor functional coupling*

The receptor activity-modifying proteins (RAMP) comprise a family of three accessory proteins that are widely distributed in cells and tissues. These proteins have been characterized for their ability to heterodimerize with the calcitonin receptor-like receptor (CL receptor) or with the calcitonin receptor (CTR) to generate different receptor pharmacological profiles [38,43]. However, the RAMPs also interact with a number of other GPCRs and receptors for the VIP family of peptides. Unlike the interaction of RAMPs with the CL receptor or the CTR, VPAC1-RAMP complexes do not show altered pharmacological behavior compared with the VPAC1 receptor alone, as

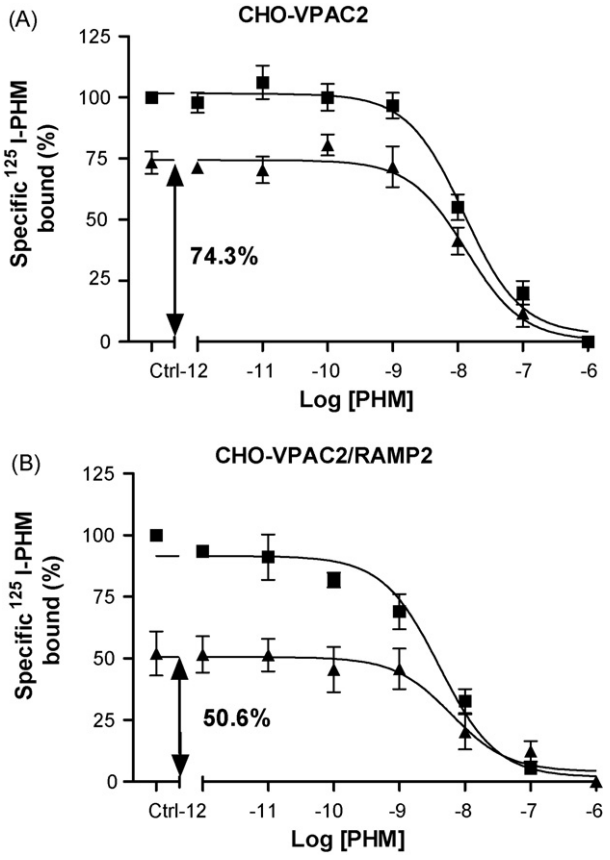

Fig. 1 – (A) Competitive inhibition in absence (■) or presence (▲) of 20 μM GppNHp of ^{125}I-PHM specific binding by PHM on CHO VPAC2 membrane. (B) Competitive inhibition of ^{125}I-PHM specific binding by PHM on CHO VPAC2 membrane transfected with RAMP2 in absence (■) or presence (▲) of 20 μM GppNHp. All values correspond to the mean ± S.E.M. calculated from three independent experiments performed in triplicate.

Table 1 – Pharmacological parameters of PHM binding sites

	IC50 ($\times 10^9$ M)	GTP Insensitivity (%)
CHO VPAC2	12.5	
CHO VPAC2 + GppNHp (20 μM)	13.5	74.3
CHO VPAC2/Ramp1	8.1	
CHO VPAC2/Ramp1 + GppNHp (20 μM)	14.9	47.3
CHO VPAC2/Ramp2	4.09	
CHO VPAC2/Ramp2 + GppNHp (20 μM)	6.21	50.6
CHO VPAC2/Ramp3	13	
CHO VPAC2/Ramp3 + GppNHp (20 μM)	17	66

Data were calculated from competitive inhibition of [^{125}I]-PHM binding by PHM obtained on CHO VPAC2 cells (Fig. 1A) and CHO VPAC2 transfected with Ramp2 (Fig. 1B), RAMP1 and RAMP3 (data not shown) with or without 20 μM GppNHp.

determined using radioligand binding in COS-7 cells [12]. However, the VPAC1-RAMP2 heterodimer displays a significant enhancement of agonist-mediated phosphoinositide hydrolysis with no change in cAMP stimulation compared with VPAC1 alone [12]. These findings represent the original demonstration of a novel consequence of RAMP-receptor interaction: the modulation of cell signaling, through a commutation of the coupling of a GPCR to different G proteins.

6.2. *The receptor activity-modifying proteins (RAMP) modulate VPAC2 sensitivity to GTP*

In continuation of the original data on the sensitivity of the VPAC2 receptor to GTP presented in the preceding section (Fig. 1A), we sought to determine whether, as for the VPAC1, VPAC2 binding properties could be modified in the presence or absence of a given RAMP protein (unpublished data). The effects of the three different RAMP proteins on the GTP sensitivity of the VPAC2 receptors was studied in stably transfected CHO cells expressing this receptor, using ^{125}I-PHM as a radiotracer. It is worth noting that these cells do not express any RAMP. PHM was chosen instead of VIP in these experiments because of its higher ability to discriminate GppNHp insensitive binding sites. Some unpublished data from our group are partly presented here. They allowed us to observe that RAMP2, but not the two other RAMPs, increased the PHM binding affinity for the VPAC2 receptor, reaching EC50 values in the nanomolar range. RAMP2 also decreased the proportion of GTP insensitive PHM binding sites in these membranes from 74 to 50%, suggesting an optimized coupling of the receptor in the presence of this accessory protein (Table 1). Data were calculated from competitive inhibition of ^{125}I-PHM binding by PHM obtained in CHO VPAC2 cell membranes (Fig. 1A) and CHO VPAC2 transfected with RAMP2 (Fig. 1B), RAMP1 and RAMP3 (data not shown) with or without 20 μM GppNHp. Of the three RAMP proteins, RAMP2 possesses the longest extracellular tail, suggesting that the N-terminal extremity of RAMP2 may represent a domain interacting with VPAC2 and modulating its affinity. This set of data suggests that fully active PHM receptors may in fact correspond to VPAC2 receptors associated with RAMP2. Such a model closely resembles that represented by the interaction between RAMPs and CRLR to generate active CGRP or adrenomedullin receptor complexes [38,43].

Taken globally, data from our studies allow the proposal that RAMP2 may be an essential component to enhance VPAC2 coupling and/or trigger a commutation of the receptor coupling with intracellular effectors. This reinforces the hypothesis that the coupling efficiency and/or specificity of VPAC2 with the intracellular signaling pathways depends not only on the receptor itself but also on its environment and on the nature of its partners present at the level of the cell membrane.

6.3. *Calmodulin or related proteins could regulate GTP sensitivity of PHI binding sites in rat brain*

Since PHI preferentially discriminates GTP-insensitive binding sites, ^{125}I-PHI was utilized as a radiotracer in binding experiments to investigate their expression in rat brain tissue, at two stages: postnatal day 2 (P2) and adult (2 months old

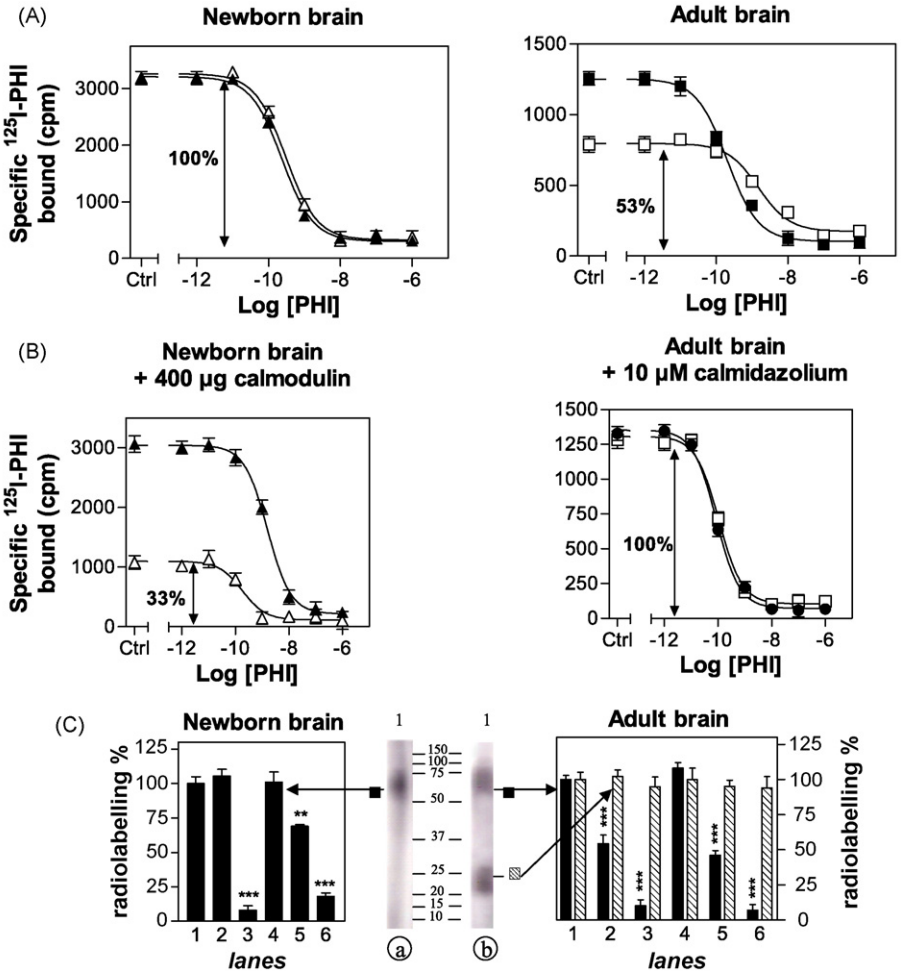

Fig. 2 – (A) Competitive inhibition of [^{125}I]-PHI binding by PHI, on newborn (left panel, ▲ and △) or adult (right panel, ■ and □) rat brain membranes, in absence (▲, ■) or presence (△, □) of 20 μM GppNHp. (B) Competitive inhibition of [^{125}I]-PHI binding by unlabeled PHI, on rat brain membrane in the absence (▲, ■) or in the presence (△, □) of 20 μM GppNHp, with 400 ng/assay of bovine brain calmodulin in newborn (left panel, ▲ and △) or with 10 μM of calmidazolium/assay in adult (right panel, ◆ and □). (C) Covalent cross-linking of newborn (a) or adult (b) rat brain membrane proteins (350 μg) with [^{125}I]-PHI (200 pM), detected by SDS-PAGE and autoradiography (only control lanes are shown). Membranes were incubated for 50 min at 20 °C with radiotracer and lane 1: no addition (CTRL); lane 2: 20 μM GppNHp; lane 3: 10^{-7} M PHI + 20 μM GppNHp; lane 4: 10^{-12} M PHI; lane 5: 10^{-10} M PHI; lane 6: 10^{-7} M PHI. Finally all samples were treated with 1 mM DSP. Two radiolabeled components A (M_r = 65,000 ± 0.030; ■) and B (M_r = 24,000 ± 0.700; □) were observed. Markers from 150 to 10 kDa are indicated. The relative molecular weights represent the means ± S.E.M. of three independent tests. Autoradiographies were quantified by densitometry analysis (Scion Image Beta 4.02, Scion Corporation) and were illustrated by histograms. Statistics were performed: $p < 0.01$ (**); $p < 0.001$ (***). Data from binding studies represent the means ± S.E.M. of three independent experiments, each performed in triplicate. For cross-linking studies, three independent experiments were effected.

rats). The overall PHI binding capacity for the same amount of brain membrane proteins was two-fold greater in P2 than in adult animals, with similar affinities at both stages (Fig. 2A). In P2 brain, the same binding capacity and affinity was observed in the presence or absence of 20 μM GppNHp (Fig. 2A, left). In adult rat brain membranes, ^{125}I-PHI binding capacity was strongly reduced (53%) in the presence of GppNHp (Fig. 2A, right) with a slight reduction of the peptide binding affinity (Fig. 2A, right). The exclusive expression of GTP-insensitive PHI binding sites in newborn rat brain suggests a crucial implication of these receptors in neurodevelopment. It has been proposed that the GTP-insensitive sites may correspond to structurally immature receptors [27], but another explanation could be an alternative coupling of same receptors to different signaling pathways. In any case, these data indicate that PHI may ensure typical functions in early development that are distinct of that of VIP, since this VIP analogue interacts more selectively than VIP to GTP-insensitive binding sites.

Several reports indicate that seven transmembrane spanning domain receptors such as the metabotropic glutamate 5 receptor [35] and the OP3 opioid receptor [60] may interact with calmodulin. Other authors have proposed that VIP

receptors could be associated with a 17–18 kDa membrane-bound calmodulin-like protein [2]. These observations led us to study the potential role of calmodulin and calmidazolium, its specific inhibitor, in the regulation of GTP-sensitivity of the selective PHI binding sites in the rat brain. In newborn brain membranes, in the presence of both 20 μM GppNHp and calmodulin at 1.6 ng/μl (a concentration giving the maximal effect), ^{125}I-PHI specific binding represented only 33% of the radiotracer specific binding (Fig. 2B, left) obtained in the presence of the nucleotide derivative alone (Fig. 2A, left). Hence, addition of calmodulin to newborn rat membranes strongly decreases the proportion of GTP-insensitive binding sites. Additionally, in adult rat brain membranes, in the presence of 20 μM GppNHp, 10 μM calmidazolium completely abolished the ^{125}I-PHI GppNHp-sensitive binding (Fig. 2B, right). In other words, the data indicate that in adult tissue, in the presence of the calmodulin inhibitor, all the ^{125}I-PHI binding sites corresponded to GppNHp-insensitive components (Fig. 2B, right), while in the absence of calmidazolium, only a fraction (53%) of the ^{125}I-PHI binding capacity was GTP-insensitive (Fig. 2A, right). Interestingly, neither calmodulin nor calmidazolium significantly modified the affinity of the ^{125}I-PHI binding sites expressed in rat brain membranes.

In our studies on rat brain membranes, covalent cross-linking experiments using ^{125}I-PHI revealed two radiolabeled components in the adult tissue, with relative molecular masses of 65 and 24 kDa while in the newborn, the 65 kDa species was only observed. In adult rat brain, labeling of the 65 kDa but not the 24 kDa species was partially abolished by 20 μM GppNHp, suggesting that the 65 kDa component could correspond to both GTP-sensitive and GTP-insensitive PHI binding sites (Fig. 2C, lane 2, right). In newborn rat brain membranes the 65 kDa uniquely observed was totally insensitive to GTP (Fig. 2C, lane 2 left). The glycosylated VIP receptor was described in the same range of molecular mass [53] in human liver and in rat brain cortex [13]. VPAC1 and VPAC2 receptors of 67 and 68 kDA, respectively, were identified in human lung membranes [11].

The additional 24 kDa component was only detected in adult rat brain expressing both GTP-sensitive and -insensitive PHI binding sites. In the rat brain cortex, a component of 18 kDa interacting with ^{125}I-VIP was detected by cross-linking [13] but its nature was not clarified. Later, a 17–18 kDa species, which bound ^{125}I-VIP with a low affinity, was purified and identified as a calmodulin-like membrane-associated protein, in the guinea pig [10,56] and in the pig [2]. In our experiments, the GTP-sensitive PHI binding capacity was totally abolished in the presence of the calmodulin inhibitor in adult brain membranes (Fig. 2B, right). On the contrary, addition of brain calmodulin to membrane preparations increased the proportion of GTP-sensitive PHI binding sites in newborn rat brain (Fig. 2B, left).

The concomitant lack of both GTP-sensitive PHI binding sites and of labeling of the 24 kDa component in adult brain membranes, strongly supports that this component is essential for the acquisition of the GTP-sensitivity of PHI binding components. The 24 kDa form observed in our experiments had pharmacological behavior similar to calmodulin, suggesting that this species could be closely related to the calmodulin-like membrane-associated 18 kDa protein previously described in the guinea pig and pig using ^{125}I-VIP cross-linking experiments [2,10,56]. Hence, it can be concluded that in the adult rat brain, either calmodulin or a related molecule could closely interact with the VPAC receptors expressed in adult rat brain membranes, which allows this 24 kDa form to be cross-linked to the radiotracer. This interaction, which leads to the generation of GTP-sensitive binding sites, could reflect an increased functional coupling of the receptors to G proteins and their associated effectors.

7. Conclusions

The data presented here are far from exhaustive, but they illustrate the complexity of the initial phenomenon involved in the action of VIP and related peptides PACAP and PHI/PHM on target cells and tissues, which is their interaction with numerous binding sites. Some of them correspond to the definition of the conventional receptors VPAC1, VPAC2 and PAC1 which have been extensively characterized structurally and functionally. However, other high affinity binding species for these peptides have been clearly identified in terms of binding properties. These entities displaying pharmacological behavior which does not correlate with that of conventional receptors are: the ''GTP-insensitive'', the high affinity ''VIP/PHI/PHM'', the ''VIP-preferring'' and the ''PHI/PHM-preferring'' binding sites. These binding sites still remain to be identified at the molecular level; however, several novel elements of knowledge reveal that they may not be as ''unconventional'' as they look, and these data may lead future investigations dedicated to their characterization.

As presented in Section 5.1, experiments from the group of Hill, using cross-linking of ^{125}I-VIP in rat brain membrane preparations, revealed 4 binding species with molecular masses ranging from 44 to 92 kDa. Only one of these, a 64 kDa species, behaves like a GppNHp-sensitive receptor, the others of course being insensitive to this nucleotide derivative [25]. This observation raises the question of the molecular nature of the other components: are they three different molecules or a same component associated with different partners? Similarly, studies from our group conducted in chicken liver, using ^{125}I-PHI cross-linking, demonstrated that two binding sites of 48 and 60 kDa were represented in this tissue. Interestingly, in these studies, the 48 kDa species was GppNHp-insensitive and displayed a high affinity for PHI while the 60 kDa component was sensitive to the nucleotide and interacted poorly with VIP. In other words, PHI discriminated a 48 kDa GppNHp-insensitive component from a 60 kDa GppNHp sensitive form [45]. Again, these two species could of course correspond to different molecules, but they may also represent different states of the same protein resulting from its association with one or another partner.

A first interesting clue is the possible interaction of GPCR with accessory proteins, leading to the formation of heterodimers. In fact, in addition to G proteins, GPCR can interact through their third intracellular loop with a number of proteins, possessing typical domains, such as PDZ motifs [7]. Important accessory proteins are the beta-arrestins, which are involved in receptor desensitization and internalization [48]. It has been proposed that these components could couple

GPCR to the MAP-kinase pathway in a G-protein independent fashion. Other proteins known to interact with GPCR and to modulate receptor activity and coupling are the RAMPs and calmodulin, or the calmodulin analogue RCP (receptor component protein) which plays a crucial role in the coupling of G proteins with some GPCR [47]. As a matter of fact, RAMP2 was shown to modulate VPAC1 and VPAC2 receptor activity. RAMP2 switches the coupling of the VPAC1 receptor without apparent alteration of the binding characteristics of this receptor. In our own experiments, VPAC2 receptors represented a good candidate to generate GTP-insensitive binding sites that are discriminated by the VIP analogue PHM, in the context of CHO cells. RAMP2 increased the binding affinity of VPAC2 for PHM and optimized the proportion of GTP-sensitive sites for this peptide, hence the coupling efficiency of the corresponding receptor. Moreover, calmodulin was able to potently induce the generation of GTP-sensitive high affinity ^{125}I-PHI binding sites in newborn rat brain membranes where 100% of the PHI binding capacity is represented by GTP-insensitive species. Hence, the same receptor may exist as a GTP sensitive or insensitive species, leading to differential coupling to intracellular signaling pathways, whether or not it interacts with an accessory protein.

Another avenue for investigation could be the analysis of allosteric interactions between VPAC1, VPAC2, PAC1 and their numerous isoforms in tissues where some of them may coexist. Utilization of bioluminescence resonance energy transfer (BRET) or fluorescence resonance energy transfer (FRET) revealed that some class I or class II GPCR can function as homo- or hetero-dimers. GPCR dimerization seems necessary to pass quality-control checkpoints of the biosynthetic pathway of GPCRs, as demonstrated for a small number of receptors that must form heterodimers to be exported properly to the plasma membrane (referred to as obligatory heterodimers). Increasing amounts of data also suggest that dimers represent the basic functional signaling unit for some members of this receptor family, the most prominent example being the $GABA_B$ receptor for the neurotransmitter gamma-aminobutyric acid (GABA) which is only fully active in a dimeric state [34]. Using constructs allowing expression of Renilla reniformis luciferase, yellow fluorescent protein, or cyan fluorescent protein at the carboxyl terminus of VPAC1, VPAC2, and secretin receptors, and performing BRET and morphologic FRET studies with all combinations, revealed that VPAC1 and VPAC2 also form constitutive oligomers with themselves and with the secretin receptor. These constructs bound their natural ligands specifically and saturably, with these agonists able to elicit full cAMP responses. The VPAC1–VPAC2 receptor hetero-oligomers reached the cell surface, where receptor interactions were clear [18,23]. It is of particular importance to verify whether such oligomerization of these receptors also occurs in native tissues, using for instance co-immunoprecipitation or co-localization by confocal microscopy.

A careful scan of the gene banks did not reveal any novel GPCR sharing significant sequence similarities with the conventional VIP and PACAP receptors. But unrelated molecules may also behave like receptors for these neuropeptides. An example illustrated in this report is represented by Atrial natriuretic receptors, particularly the ANP-C subtype which have been demonstrated to generate high affinity binding sites for VIP and related peptides. Furthermore, the PAC1 receptor, and to a much lesser extent the VPAC1 or VPAC2 genes, give rise to a number of isoforms whose pharmacological and functional properties are not yet fully understood. Some of these isoforms, like the hop2 variant or those lacking the domains corresponding to exons 5 and 6 of the PAC1 receptor gene have been reported to behave like VPAC receptors: they share quite similar affinities for both VIP and PACAP. Hence, there is an urgent need for detailed pharmacological studies based on expression of these different isoforms in transfected cells, in order to analyze whether some of them could reproduce a pharmacological behavior of certain non conventional VIP or PACAP binding sites. A precise anatomical analysis of the distribution of these variants in different tissues may allow a comparison of their pattern of expression with that of the unconventional binding sites. Furthermore, it should be also of importance to analyze whether these isoforms could oligomerize each other in cell membranes.

To conclude, the road is still long to unveil the molecular principles that generate the complex set of binding sites which is involved in the interaction of VIP and related peptides PACAP and PHI/PHM with target cells. Recent progress concerning the knowledge of the VPAC1, VPAC2 and PAC1 receptors, including the discovery of numerous isoforms of these molecules, the possible oligomerization of these components and their interaction with accessory proteins, allow a glimpse of the numerous combinations in which they could be involved. This opens a wide field of investigation that may lead to unexpected and spectacular discoveries concerning the pharmacological and functional properties of these receptor/accessory proteins complexes and their utilization as therapeutic targets, through the development of more selective and efficient peptide derivatives.

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REFERENCES

[1] Amiranoff B, Laburthe M, Rosselin G. Differential effects of guanine nucleotides on the first step of VIP and glucagon action in membranes from liver cells. Biochem Biophys Res Commun 1980;96:463–8.

[2] Andersson M, Carlquist M, Maletti M, Marie J-C. Simultaneous solubilization of high-affinity receptors for VIP and glucagon and of a low-affinity binding protein for VIP, shown to be identical to calmodulin. FEBS 1993;318: 35–40.

[3] Arimura A. Perspectives on pituitary adenylate cyclase activating polypeptide (PACAP) in the neuroendocrine, endocrine, and nervous systems. Jpn J Physiol 1998;48: 301–31.
[4] Abad C, Gomariz RP, Waschek JA. Neuropeptide mimetics and antagonists in the treatment of inflammatory disease: focus on VIP and PACAP. Curr Top Med Chem 2006;6: 151–63.
[5] Adamou JE, Aiyar N, Van Horn S, Elshourbagy NA. Cloning and functional characterization of the human vasoactive intestinal peptide (VIP)-2 receptor. Biochem Biophys Res Commun 1995;209:385–92.
[6] Akiho H, Chijiiwa Y, Okabe H, Harada N, Nawata H. Interaction between atrial natriuretic peptide and vasoactive intestinal peptide in guinea pig cecal smooth muscle. Gastroenterology 1995;109:1105–12.
[7] Bockaert J, Roussignol G, Becamel C, Gavarini S, Joubert L, Dumuis A, et al. GPCR-interacting proteins (GIPs): nature and functions. Biochem Soc Trans 2004;32:851–5.
[8] Bokaei PB, Ma XZ, Byczynski B, Keller J, Sakac D, Fahim S, et al. Identification and characterization of five-transmembrane isoforms of human vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide receptors. Genomics 2006;88:791–800.
[9] Brabet P, Diriong S, Journot L, Bockaert J, Taviaux S. Localization of the human pituitary adenylate cyclase-activating polypeptide receptor (PACAP1-R) gene to 7p15-p14 by fluorescence in situ hybridization. Genomics 1996;38:100–2.
[10] Brugger CH, Stallwood D, Paul S. of a low molecular mass vasoactive intestinal peptide binding protein. J Biol Chem 1991;266:18358–62.
[11] Busto R, Carrero I, Guijarro LG, Solano RM, Zapatero J, Noguerales F, et al. Expression, pharmacological, and functional evidence for PACAP/VIP receptors in human lung. Am J Physiol 1999;277:L42–8.
[12] Christopoulos A, Christopoulos G, Morfis M, Udawela M, Laburthe M, Couvineau A, et al. Novel receptor partners and function of receptor activity-modifying proteins. J Biol Chem 2003;278:3293–7.
[13] Couvineau A, Gammeltoft S, Laburthe M. Molecular characteristics and peptide specificity of vasoactive intestinal peptide receptors from rat cerebral cortex. J Neurochem 1986;47:1469–75.
[14] Couvineau A, Rouyer-Fessard C, Maoret JJ, Gaudin P, Nicole P, Laburthe M. Vasoactive intestinal peptide (VIP)1 receptor. Three nonadjacent amino acids are responsible for species selectivity with respect to recognition of peptide histidine isoleucineamide. J Biol Chem 1996;271:12795–800.
[15] Debaigt C, Meunier AC, Goursaud S, Montoni A, Pineau N, Couvineau A, et al. Expression and GTP sensitivity of peptide histidine isoleucine high-affinity-binding sites in rat. Ann NY Acad Sci 2006;1070:215–9.
[16] Dejda A, Sokolowska P, Nowak JZ. Neuroprotective potential of three neuropeptides PACAP, VIP and PHI. Pharmacol Rep 2005;57:307–20.
[17] Dufes C, Alleaume C, Montoni A, Olivier JC, Muller JM. Effects of the vasoactive intestinal peptide (VIP) and related peptides on glioblastoma cell growth in vitro. J Mol Neurosci 2003;21:91–102.
[18] George SR, O'Dowd BF, Lee SP. G-protein-coupled receptor oligomerization and its potential for drug discovery. Nat Rev Drug Discov 2002;808–20.
[19] Goursaud S, Pineau N, Becq-Giraudon L, Gressens P, Muller JM, Janet T. Human H9 cells proliferation is differently controlled by vasoactive intestinal peptide or peptide histidine methionine: implication of a GTP-insensitive form of VPAC1 receptor. Neuroimmunology 2005;158: 94–105.
[20] Gozes I, Furman S. VIP and drug design. Curr Pharm Des 2003;9:483–94.
[21] Gressens P, Hill JM, Gozes I, Fridkin M, Brenneman DE. Growth factor function of vasoactive intestinal peptide in whole cultured mouse embryos. Nature 1993;362:155–8.
[22] Guidone G, Muller D, Vogt K, Mukhopadhyay AK. Characterization of VIP and PACAP receptors in cultured rat penis corpus cavernosum smooth muscle cells and their interaction with guanylate cyclase-B receptors. Regul Pept 2002;108(2/3):63–72.
[23] Harikumar KG, Morfis MM, Lisenbee CS, Sexton PM, Miller LJ. Constitutive formation of oligomeric complexes between family B G protein-coupled vasoactive intestinal polypeptide and secretin receptors. Mol Pharmacol 2006;69(1):363–73.
[24] Harmar AJ, Arimura A, Gozes I, Journot L, Laburthe M, Pisegna JR, et al. International Union of Pharmacology. XVIII. Nomenclature of receptors for vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide. Pharmacol Rev 1998;50:265–70.
[25] Hill JM, Harris A, Hilton-Clarke DI. Regional distribution of guanine nucleotide-sensitive and guanine nucleotide-insensitive vasoactive intestinal peptide receptors in rat brain. Neuroscience 1992;48:925–32.
[26] Hill JM, Agoston DV, Gressens P, McCune SK. Related articles, distribution of VIP mRNA and two distinct VIP binding sites in the developing rat brain: relation to ontogenic events. J Comp Neurol 1994;342:186–205.
[27] Laburthe M, Breant B, Rouyer-Fessard C. Molecular identification of receptors for vasoactive intestinal peptide in rat intestinal epithelium by covalent cross-linking. Evidence for two classes of binding sites with different structured and functional properties. Eur J Biochem 1984;139:181–7.
[28] Laburthe M, Couvineau A, Marie JC. VPAC receptors for VIP and PACAP. Receptors Channels 2002;8:137–53.
[29] Langer I, Vertongen P, Perret J, Waelbroeck M, Robberecht P. Lysine 195 and aspartate 196 in the first extracellular loop of the VPAC1 receptor are essential for high affinity binding of agonists but not of antagonists. Neuropharmacology 2003;44:125–31.
[30] Langer I, Langlet C, Robberecht P. Effect of inactivating mutations on phosphorylation and internalization of the human VPAC2 receptor. J Mol Endocrinol 2005;34:405–14.
[31] Lelievre V, Pineau N, Du J, Wen CH, Nguyen T, Janet T, et al. Differential effects of peptide histidine isoleucine (PHI) and related peptides on stimulation and suppression of neuroblastoma cell proliferation. A novel VIP-independent action of PHI via MAP kinase. J Biol Chem 1998;273: 19685–90.
[32] Lelievre V, Hu Z, Ioffe Y, Byun JY, Flores A, Seksenyan A, et al. Paradoxical antagonism of PACAP receptor signaling by VIP in *Xenopus* oocytes via the type-C natriuretic peptide receptor. Cell Signal 2006;18:2013–21.
[33] Lutz EM, Ronaldson E, Shaw P, Johnson MS, Holland PJ, Mitchell R. Characterization of novel splice variants of the PAC(1) receptor in human neuroblastoma cells: consequences for signaling by VIP and PACAP. Mol Cell Neurosci 2006;31:193–209.
[34] Milligan G. G-protein-coupled receptor heterodimers: pharmacology, function and relevance to drug discovery. Drug Discov Today 2006;11:541–9.
[35] Minakami R, Jinnai N, Sugiyama H. Phosphorylation and calmodulin binding of the metabotropic glutamate receptor subtype 5 (mGluR5) are antagonistic in vitro. J Biol Chem 1997;272:20291–8.
[36] Moody TW, Chan D, Fahrenkrug J, Jensen RT. Neuropeptides as autocrine growth factors in cancer cells. Curr Pharm Des 2003;9:495–509.

[37] Moody TW, Hill JM, Jensen RT. VIP as a trophic factor in the CNS and cancer cells. Peptides 2003;24:163–77.
[38] Morfis M, Christopoulos A, Sexton PM. RAMPs: 5 years on, where to now? Trends Pharmacol Sci 2003;24:596–601.
[39] Muller JM, Lelievre V, Becq-Giraudon L, Meunier AC. VIP as a cell-growth and differentiation neuromodulator role in neurodevelopment. Mol Neurobiol 1995;10:115–34.
[40] Muller JM, Philippe M, Chevrier L, Heraud C, Alleaume C, Chadeneau C. The VIP-receptor system in neuroblastoma cells. Regul Pept 2006;137:34–41.
[41] Murthy KS, Zhang KM, Jin JG, Grider JR, Makhlouf GM. VIP-mediated G protein-coupled Ca^{2+} influx activates a constitutive NOS in dispersed gastric muscle cells. Am J Physiol 1993;265:G660–71.
[42] Murthy KS, Teng BQ, Zhou H, Jin JG, Grider JR, Makhlouf GM. $G(i-1)/G(i-2)$-dependent signaling by single-transmembrane natriuretic peptide clearance receptor. Am J Physiol Gastrointest Liver Physiol 2000;278:G974–80.
[43] Parameswaran N, Spielman WS. RAMPs: The past, present and future. Trends Biochem Sci 2006;31:631–8.
[44] Paul S, Chou J, Kubota E. High affinity peptide histidine isoleucine-preferring receptors in rat liver. Life Sci 1987;41:2373–80.
[45] Pineau N, Lelievre V, Goursaud S, Hilairet S, Waschek JA, Janet T, et al. The polypeptide PHI discriminates a GTP-insensitive form of VIP receptor in liver membranes. Neuropeptides 2001;35:117–26.
[46] Pilzer I, Gozes I. VIP provides cellular protection through a specific splice variant of the PACAP receptor: a new neuroprotection target. Peptides 2006;27:2867–76.
[47] Prado MA, Evans-Bain B, Dickerson IM. Receptor component protein (RCP): a member of a multi-protein complex required for G-protein-coupled signal transduction. Biochem Soc Trans 2002;30:460–4.
[48] Rajagopal K, Lefkowitz RJ, Rockman HA. When 7 transmembrane receptors are not G protein-coupled receptors. J Clin Invest 2005;115:2971–4.
[49] Ramirez-Cardenas R, Prieto JC, Guerrero JM, Goberna R. Guanyl nucleotide regulation of vasoactive intestinal peptide interaction with rat liver membranes. Rev Esp Fisiol 1981;37:9–16.
[50] Rangon CM, Goursaud S, Medja F, Lelievre V, Mounien L, Husson I, et al. VPAC2 receptors mediate vasoactive intestinal peptide-induced neuroprotection against neonatal excitotoxic brain lesions in mice. J Pharmacol Exp Ther 2005;314:745–52.
[51] Rangon CM, Dicou E, Goursaud S, Mounien L, Jegou S, Janet T, et al. Mechanisms of VIP-induced neuroprotection against neonatal excitotoxicity. Ann NY Acad Sci 2006;1070:512–7.
[52] Reubi JC. Peptide receptors as molecular targets for cancer diagnosis and therapy. Endocr Rev 2003;24:389–427.
[53] Rodriguez-Henche N, Rodriguez-Pena MS, Guijarro LG, Prieto JC. Characterization of vasoactive intestinal peptide receptors in human liver. Biochim Biophys Acta 1994;1221:193–8.
[54] Said SI. Vasoactive intestinal peptide. J Endocrinol Invest 1986;9:191–200.
[55] Sreedharan SP, Huang JX, Cheung MC, Goetzl EJ. Structure, expression, and chromosomal localization of the type I human vasoactive intestinal peptide receptor gene. Proc Natl Acad Sci USA 1995;92:2939–43.
[56] Stallwood D, Brugger CH, Baggenstoss BA, Stemmer PM, Shiraga H, Landers DF, et al. Identity of a membrane-bound vasoactive intestinal peptide-binding protein with calmodulin. J Biol Chem 1992;267:19617–21.
[57] Tan YV, Couvineau A, Murail S, Ceraudo E, Neumann JM, Lacapere JJ, et al. Peptide agonist docking in the N-terminal ectodomain of a class II G protein-coupled receptor, the VPAC1 receptor. Photoaffinity, NMR, and molecular modeling. J Biol Chem 2006;281:12792–8.
[58] Tse DL, Pang RT, Wong AO, Chan SM, Vaudry H, Chow BK. Identification of a potential receptor for both peptide histidine isoleucine and peptide histidine valine. Endocrinology 2002;143:1327–36.
[59] Vaudry D, Gonzalez BJ, Basille M, Yon L, Fournier A, Vaudry H. Pituitary adenylate cyclase-activating polypeptide and its receptors: from structure to functions. Pharmacol Rev 2000;52:269–324.
[60] Wang D, Sadee W, Quillan JM. Calmodulin binding to G protein-coupling domain of opioid receptors. J Biol Chem 1999;274:22081–8.
[61] Waschek JA. Vasoactive intestinal peptide: an important trophic factor and developmental regulator? Dev Neurosci 1995;17:1–7.
[62] Wettschureck N, Offermanns S, Mammalian. G proteins and their cell type specific functions. Physiol Rev 2005;85:1159–204.
[63] Zhou H, Huang J, Murthy KS. Molecular cloning and functional expression of a VIP-specific receptor. Am J Physiol Gastrointest Liver Physiol 2006;291:728–34.