# Expression and GTP Sensitivity of Peptide Histidine Isoleucine High-Affinity-Binding Sites in Rat

COLIN DEBAIGT,[a] ANNIE-CLAIRE MEUNIER,[a] STEPHANIE GOURSAUD,[a] ALICIA MONTONI,[a] NICOLAS PINEAU,[a] ALAIN COUVINEAU,[b] MARC LABURTHE,[b] JEAN-MARC MULLER,[a] AND THIERRY JANET[a]

[a]*IPBC CNRS-UMR 6187 Pôle Biologie Santé, 86022 Poitiers Cedex, France*

[b]*INSERM U683, Faculté de Médecine X. Bichat, 75018 Paris, France*

**ABSTRACT: High-affinity-binding sites for the vasoactive intestinal peptide (VIP) analogs peptide histidine/isoleucine-amide (PHI)/carboxyterminal methionine instead of isoleucine (PHM) are expressed in numerous tissues in the body but the nature of their receptors remains to be elucidated. The data presented indicate that PHI discriminated a high-affinity guanosine 5′-triphosphate (GTP)-insensitive-binding subtype that represented the totality of the PHI-binding sites in newborn rat tissues but was differentially expressed in adult animals. The GTP-insensitive PHI/PHM-binding sites were also observed in CHO cells over expressing the VPAC2 but not the VPAC1 VIP receptor.**

**KEYWORDS: VPAC1 and VPAC2 receptors; GTP-sensitivity; PHI/PHM**

## INTRODUCTION

The peptide histidine/isoleucine-amide (PHI) and its human counterpart with a carboxyterminal methionine instead of isoleucine (PHM) derive from the same protein precursor as vasoactive intestinal peptide (VIP). They are considered like moderate-to-weak agonists with a modest affinity toward the well-known VPAC1 and VPAC2 forms of VIP receptors. However, utilization of $^{125}$I-PHI or PHM as radiotracers, revealed that high-affinity receptors for these peptides are expressed in some tissues, such as liver, where we demonstrated that PHI discriminated a guanosine 5′-triphosphate (GTP)-insensitive subset of VIP receptors, corresponding to a 48-kDa molecular species, distinct from the 65-kDa GTP-sensitive receptor.[1] Other data also support the

Address for correspondence: Thierry Janet, IPBC CNRS-UMR 6187 Pôle Biologie Santé, 40 avenue du Recteur Pineau, 86022 Poitiers Cedex, France. Voice: 33-5-49-45-40-91; fax: 33-5-49-45-39-76.
e-mail: thierry.janet@univ-poitiers.fr

**Ann. N.Y. Acad. Sci. 1070: 215–219 (2006).** 
**doi: 10.1196/annals.1317.017**

idea that different classes of PHI receptors could be represented, especially in liver where PHI-preferring and bivalent PHI/VIP receptors appear to be expressed.[2] Differences were observed in the dose-dependent stimulation of adrenocorticotropic hormone (ACTH) and corticosterone released in response to VIP or PHI, suggesting that these two peptides may act on different sets of receptors.[3] A VIP receptor antagonist thought to bind with equal affinities to all known VIP receptors, inhibited the effects of VIP but not those of PHI on these hormonal secretion.[4] Our group reported specific effects of PHI that suppressed neuroblastoma cell proliferation through an inhibition of MAP kinase activity, while VIP or pituitary adenylate cyclase-activating polypeptide (PACAP) inhibited cell proliferation through a PKA-dependent mechanism.[5] The question of the molecular nature of the receptor complexes corresponding to the high-affinity PHI/PHM-binding sites remains, however, unanswered: are they subsets of the VIP/PACAP receptors with distinct coupling properties that renders them, at least partially, insensitive to guanyl nucleotides or are they unknown components that remain to be characterized? It was long established in early reports on $^{125}$I-VIP-binding studies that part of the VIP-binding sites expressed in the liver were sensitive to GTP.[6,7] The GTP-insensitive receptors appear to be expressed very early in the rat embryo (E9,5) and they may play a key role in the development of the central nervous system (CNS) and of the somites.[8] It was reported that a VIP derivative (stearyl-norleucine 17 VIP) could discriminate a subset of GTP-insensitive VIP-binding sites in the CNS.[9] The data presented here allow to precise further the knowledge of the expression of the high-affinity PHI/PHM-binding sites and the regulation of their GTP sensitivity.

## MATERIALS AND METHODS

### *Membrane Preparations*

Plasma membranes were obtained from freshly excised rat hearts and livers or from cultured CHO cells stably expressing VPAC1 and VPAC2 receptors[10] (generous gift from A. Couvineau and coll., Neuroendocrinology and Cell Biology, INSERM U410, Faculté de Médecine, Xavier Bichat, 75018 Paris, France). Samples were prepared following a previously described protocol.[11] Protein content was determined with a Bradford assay (BioRad Protein Assay Dye Reagent Concentrate, BioRad, Munich, Germany) and BSA as a standard. Membranes aliquots were then stored at $-80^{\circ}$C until use.

### *Peptides $^{125}$I-Iodination*

For binding experiments, PHI or PHM was radioiodinated using a previously described chloramine-T technique[12] with slight modifications.[1]

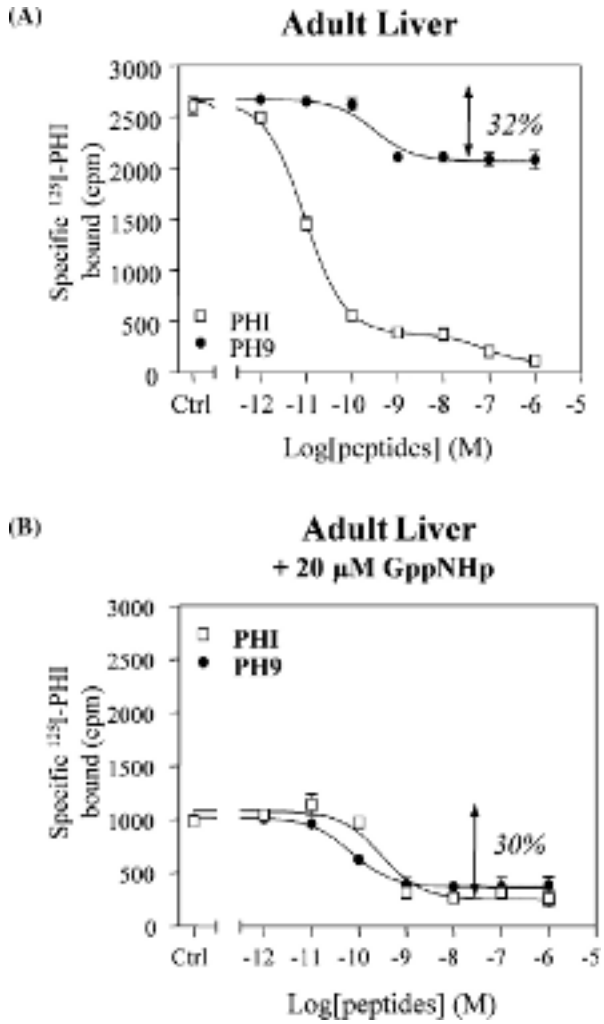


**FIGURE 1.** Competitive inhibition of $^{125}$I-PHI binding by PHI or PH9 in the absence (**A**) or in the presence (**B**) of 20 μM GppNHp in adult liver rat tissue membranes. Membranes were incubated for 50 min at 20°C with $^{125}$I-PHI (50 pM). Peptides are at indicated concentrations. Data represent the means ± SEM of three independent experiments, each performed in triplicate.

### *Receptor-Binding Studies*

The binding reactions were conducted at 20°C for 50 min, a time at which equilibrium was reached. Each radiotracer $^{125}$I-PHI or $^{125}$I-PHM in the absence or in the presence of 20 μM GppNHp, a nonhydrolyzable GTP analog, was incubated according to conditions previously reported.[1,6,7] Radioactivity was

measured in a γ-counter. In all experiments, specific binding corresponded to the difference between total binding, obtained in the absence of unlabeled PHI or PHM (according to the tracer used) and nonspecific binding, obtained in the presence of $10^{-6}$ M of PHI or PHM.

## RESULTS AND DISCUSSION

The existence of a specific PHI/PHM high-affinity receptor was first suggested in rat liver[2] but not clearly demonstrated to date. Such high-affinity-binding sites were later characterized in newborn chicken liver and described as partially insensitive to GTP.[1] GTP-insensitive-binding sites expression was also highly detected during early embryos neurogenesis and their distribution changed throughout the CNS development.[8]

In adult rat liver membranes, comparison of the total $^{125}$I-PHI-binding capacity, in the absence or in the presence of GppNHp (FIG. 1 A versus FIG. 1 B), reveals that the specific binding of $^{125}$I-PHI in the presence of the nonhydrolyzable GTP analog (FIG. 1 B) represented only 30% of the total radiotracer binding observed in control membranes (FIG. 1 A).

This result indicated that in adult rat liver 30% of the PHI-binding sites were GTP insensitive. The GTP-insensitive-binding site is selectively discriminated by PHI/PHM and more efficiently by a peptide called PH9, isolated in our laboratory. In fact, this peptide only inhibited the specific binding of $^{125}$I-PHI on GTP-insensitive-binding sites even in the absence of GppNHp (FIG. 1A).

Screening by expression cloning of a cDNA liver library with the aim to identify the PHI/PHM receptor(s) led to the isolation of a cDNA (clone6), which confers high-affinity $^{125}$I-PHI-binding properties when transfected in COS7 cells.

Binding experiments performed in different newborn rat tissues indicated that only GTP-insensitive high-affinity PHI-binding sites were detected. In adult rat, representation of GTP-insensitive PHI-binding sites varies among tissues. For instance, a majority of sites were GTP insensitive in heart, while in liver, only 31% were detected (TABLE 1).

**TABLE 1. Expression of GTP-insensitive-binding sites in different rat tissues and CHO cells stably tranfected with VPAC1 or VPAC2 receptors**

| | | Binding of $^{125}$I-PHI/$^{125}$I-PHM % of GppNHp insensitivity |
|---|---|---|
| Liver | new born | 97% |
| | Adult | 31% |
| Heart | new born | 100% |
| | Adult | 100% |
| Cells | CHO/VPAC1 | 0% |
| | CHO/VPAC2 | 80% |

In CHO cells stably expressing VPAC1 and VPAC2 receptors[10] results indicated that only VPAC2 receptor presented intrinsic GTP insensitivity compared to VPAC1 receptor (TABLE 1).

In a recent review,[13] VPAC receptors interactions with potential PDZ motif-containing proteins and with receptor activity-modifying proteins (RAMPs) were highlighted. These observations led us to study the potential role of these proteins in the regulation of GTP sensitivity of specific PHI-binding sites.

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