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The polypeptide PHI discriminates a GTP-insensitive form of vip receptor in liver membranes

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Summary In early reports on ^{125}I-VIP binding experiments in liver membranes, it has been proposed that, the VIP binding sites were partially sensitive to GTP. Here we confirm that the VIP binding sites of chicken liver membranes consisted mainly in bivalent VIP/PACAP receptors and that about 50% of the ^{125}I-VIP binding capacity was 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 suggested that GTP-insensitive VIP receptors may correspond to a subclass of high-affinity PHI receptors. Cross-linking of ^{125}I-VIP or ^{125}I-PHI to their receptors, revealed 2 components of 48 and 60 kDa. The radiolabelling of the 60 kDa component was strongly affected by increasing concentrations of the GTP analogue but was modestly abolished by an excess of PHI. Conversely, the radiolabelling 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 48 kDa component expressed in chicken liver membranes display the properties of a GTP-insensitive VIP/PHI receptor that can be pharmacologically discriminated from the GTP-sensitive 60 kDa form, through its much higher affinity for PHI. © 2001 Harcourt Publishers Ltd

INTRODUCTION

The ubiquitous 28-amino-acid Vasoactive Intestinal Polypeptide (VIP), belongs to the so-called secretin family of bioactive polypeptides, which also includes glucagon, peptide histidine/isoleucine-amide (PHI) and its human counterpart PHM (with a carboxyterminal methionine instead of isoleucine) that derive from the same protein precursor than VIP and the more recently characterized pituitary adenylate-cyclase activating polypeptide (PACAP). The neuropeptides VIP and PACAP are mediators in a wide array of physiological regulations of virtually all vital functions in the body (Handelmann, 1985). Besides their biological roles as neuromodulators, VIP and/or PACAP have also been demonstrated to regulate cell growth and/or differentiation in non cancer and cancer cell types from tissues as diverse as intestine, lung, lymphocytes, brain or sympathetic ganglia. Potent trophic effects of VIP have also been observed in whole explanted embryos and involvement of these neuropeptides during neurodevelopment, especially for PACAP, in patterning and neurogenesis (Muller et al., 1995; Moody and Cuttitta, 1993; Gozes and Brenneman, 1989; Gressens et al., 1993; Waschek et al., 1998) has been proposed.

At least three different receptors for VIP, PACAP and PHI/PHM have been extensively characterized, in terms of sequence, pharmacological profile, tissue expression and associated signalling pathways:

– the 'PACAP-preferring receptor', called PAC1 (Harmar et al., 1998), that binds more exclusively PACAP, with modest affinity for VIP and practically no interaction with PHI/PHM (Haschimoto et al., 1993). At least 7 splice variants of this receptor have been reported that differ in their ability to bind PACAP analogues and in their coupling to signalling pathways (Journot et al., 1995).

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– the polyvalent VPAC1 and VPAC2 receptors that recognize with a similar high-affinity (in the nanomolar range) VIP and PACAP, and PHI/PHM with at least a 10-fold lower affinity (Gourlet et al., 1998).

Analysis of functional properties of these receptors overexpressed in transfected cells, confirmed that both VPAC1 and VPAC2 are at least positively coupled to adenylatecyclase and that PHI/PHM peptides are low affinity agonists on both polyvalent receptors, reinforcing the statement proposed long ago that PHI/PHM and VIP interact with same receptors (Gourlet et al., 1998). However, limited data also support the 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 (Paul et al., 1987). Specific PHI receptors that were resistant to ligand desensitization have also been characterized in gastric muscle cells, beside VIP-specific and bivalent VIP/PHI receptors (Murthy et al., 1993). Differences in the dose-dependent stimulation of ACTH and corticosterone released in response to VIP or PHI, after administration of these peptides in the paraventricular nucleus of the hypothalamus have also been reported, suggesting that these 2 peptides may act on different sets of receptors (Nowak and Malendowicz, 1998). In the same studies, 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 hormones secretion (Alexander and Sander, 1995). More recently, our group reported specific effects of PHI that suppressed neuroblastoma cell proliferation through an inhibition of MAP kinase activity, while VIP and PACAP inhibited cell proliferation through a PKA-dependent mechanism. In summary, the data suggest that PHI/PHM may allow to discriminate a subpopulation of high affinity PHI/PHM receptors. In other terms, the peptides PHI/PHM could allow to reveal a partition of the VIP receptors into subpopulations with potentially distinct pharmacological and functional properties (Lelievre et al., 1998).

A precise analysis of the potential relationships that may link the high affinity PHI/PHM binding sites and the VIP/PACAP receptors, remains however unprecedented. We thus decided to undertake this puzzling study in liver, an homogeneous tissue where we as well as other groups, demonstrated the presence of an high VIP binding capacity (Meunier et al., 1992). The pharmacological and biochemical properties of the receptors were assessed by means of radioiodinated VIP or PHI binding or cross-linking experiments. In order to further refine the pharmacological definition of these receptors, we also utilized the non hydrolysable GTP analogue GppNHp, that allows to assess the functional coupling state of the receptors with the G-proteins. The data lead to the proposal that PHI disciminates a GTP-insensitive form of polyvalent VIP/PACAP receptors that may correspond to a disctinct molecular entity.

MATERIALS AND METHODS

Membranes preparation

Plasma membranes were obtained from freshly excised four-days chicken livers. Tissues were homogeneized using a teflon-glass Potter homogeneizer in 10 mM Tris (pH 8), 0.32 M sucrose, 5 mM $MgCl_2$, containing a mixture of proteinase inhibitors: 1 mM PMSF, 1 mg/ml bacitracin, 2 mM ortho-phenanthroline and 0.2 mM antipain. The homogenate was centrifuged at 800 g for 20 min at 4°C. The collected supernatant was centrifuged at 4°C for 90 min at 27,000 g. The resulting pellet was resuspended in the same buffer, centrifuged at 27,000 g for 90 min at 4°C. The final pellet was resuspended in 20 mM Tris (pH 8) containing the same mixture of proteinase inhibitors. Protein content was determined using the Bradford assay (Biorad). Aliquots of membrane preparations were kept at – 80°C.

Peptides radioiodination

For binding experiments, VIP, PACAP27 and PHI were radioiodinated using a previously described chloramine-T technique (Martin et al., 1986) with slight modifications. Briefly, 0.5 mCi of a ^{125}I-Na solution (about 5 µl) were mixed to 15 µl of a 10^{-4} M aqueous solution of VIP, PACAP27 or PHI (Neosystem, France). The reaction was initiated by adding 2 mg/ml of chloramine-T in a 0.2 M sodium phosphate buffer (pH 7.6), then stopped after a 1 min incubation at room temperature with 20 µl of sodium metabisulfite (2 mg/ml in a 0.2 M sodium phosphate buffer pH 7.6). The radioiodination mix was first eluted from C_{18} SEP PAK column (Waters Associates), with 85% acetonitrile in H_2O-0.1% TFA in order to remove the free radioiodine. The sample containing radioiodinated peptides was separated by reversed phase HPLC (Spectraphysics), using a 5 µm VYDAC C18 column (Interchrom, France). Elution was conducted for 32 min with a 0–85% linear gradient of acetonitrile in H_2O-0.1% TFA. Fractions corresponding to the monoiodinated forms (specific activity 2200 mCi/mmol) of VIP, PACAP-27 or PHI typically giving high specific binding were pooled, acetonitrile was evaporated under nitrogen then the resulting sample divided into aliquots was stored at – 20°C.

Receptor binding studies

The binding reactions (250 µl) were conducted at 20°C for 50 min, a time at which equilibrium was reached, as

assessed in preliminary time-course binding experiments (not shown). Each radiotracer ^{125}I-PACAP, ^{125}I-PHI or ^{125}I-VIP was incubated according to conditions reported by others (Hill et al., 1992; Amiranoff et al., 1980), at a concentration of about 30pM in the presence of 100 μg membrane proteins in binding buffer (10 mM HEPES, 130 mM NaCl, 4.7 nM KCl, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM EDTA, 0.1% bacitracine, 1% BSA), in the presence or in the absence of increasing concentrations of unlabelled peptides and of 20 μM GppNHp. The reaction was terminated by filtration under reduced pressure through Whatman GF/C glass-fiber filters (Polylabo, France) pretreated for 30 min with an aqueous solution of 1% polyethyleneimine, 0.1% casein. The filters were washed twice with 150 mM Tris, 5 mM $MgCl_2$, 0.1% caseine. Radioactivity retained on filters was measured in a LKB-Wallacr γ-counter. In all these experiments, specific binding corresponded to the difference between total binding, obtained in the absence of unlabelled VIP, PACAP27 or PHI (according to the tracer utilized) and non specific binding, obtained in the presence of 10^{-7} M of VIP, PACAP27 or PHI.

Kinetic binding experiments with ^{125}I-PHI radiotracer were carried out in similar conditions, for the periods of time indicated in the corresponding figures. Data were analysed according to a rectangular hyperbola equation: $B = (Be.t)/(t + t_{1/2})$, where B is the specific binding for each incubation time, Be is the binding at equilibrium and $t_{1/2}$ is the incubation time allowing to attain 50% of Be. Data were linearized by a pseudo-first order equation: $Ln(Be/(Be-B)) = Kobs.t$, where Kobs is the observed association constant.

Cross-linking of radioiodinated VIP or PHI to liver membrane receptors

Binding of ^{125}I-VIP was performed under agitation by incubating membrane preparations (200 μg proteins) with 300 pM ^{125}I-VIP or 400 pM ^{125}I-PHI in the presence or in the absence of specified additions, using the same method described above. Membranes were recovered by centrifugation at 80,000 g for 20 min at 4°C.

Covalent cross-linking of bound ^{125}I-VIP to its receptors (Muller et al., 1985) was obtained by incubating membrane pellets resuspended in 250 μl solution of 60 mM HEPES (pH 8.1), 160 mM NaCl, 150 μM PMSF, 300 μM orthophenanthroline and 2 mM BSOCOES (Pierce, France), for 30 min at room temperature. Reaction was stopped by addition of an excess of ammonium acetate at a final concentration of 20 mM then centrifuged at 80,000 g for 20 min at 4°C. The resulting pellets were resuspended in 40 μl of 5-fold concentrated SDS-sample buffer (Tris 0.05 M, SDS 2.5%, glycerol 10%, pH 6.8) without reducing agent. Electrophoresis in SDS-polyacrylamide gels (10%) was run according to Laemmli (Laemmli, 1970), under non-reducing conditions. After electrophoresis, the gels were stained with Coomassie blue R-250 (Sigma, France), dried and autoradiographed using a Kodak BioMax MS film and enhancing screens, for an average of 15 days at − 80°C. The autoradiographic data were quantified by densitometric analysis (Molecular Dynamics™) according to manufacturer instructions.

RESULTS

Binding of ^{125}I-PACAP 27, ^{125}I-VIP or ^{125}I-PHI in liver membranes; pharmacological profiles

Expression of the PACAP, PHI and VIP binding sites in liver membranes was studied by means of experiments of competitive displacement of ^{125}I-PACAP27 (Fig 1A), ^{125}I-VIP (Fig 1B) or ^{125}I-PHI (Fig 1C) by unlabeled PACAP27, VIP, PHI, secretin or glucagon. Fitting of the data to the Hill equation by non-linear regression computed analysis (GraphPAD™ PRISM), allowed to obtain for each radioligand a pharmacological profile, i.e. the order of IC_{50} values (relative affinity) of the unlabelled polypeptides for each subset of radioligand binding site. These parameters summarized in Table 1 reveal that unlabeled PACAP27, VIP or PHI, but not secretin or glucagon, interacted with each radiotracer's binding sites. The IC_{50} values were generally in the nanomolar range or better, corresponding to high-affinity interaction of PACAP27, VIP or PHI with the radioligand binding sites, except with unlabeled PHI that

Table 1

	^{125}I-VIP displacement		^{125}I-P27 displacement		^{125}I-PHI displacement	
Ligands	**IC_{50} ± SEM (nM)**	**Hill slope ± SEM**	**IC_{50} ± SEM (nM)**	**Hill slope ± SEM**	**IC_{50} ± SEM (nM)**	**Hill slope ± SEM**
VIP	0.13 ± 0.1	0.87 ± 0.13	0.5 ± 0.1	1 ± 0.2	0.38 ± 0.3	0.97 ± 0.1
PACAP-27	0.79 ± 0.1	0.97 ± 0.18	1.2 ± 0.2	0.6 ± 0.1	0.68 ± 0.2	0.80 ± 0.1
PHI	28.60 ± 0.39	0.70 ± 0.3	33 ± 0.3	0.6 ± 0.2	0.54 ± 0.3	0.67 ± 0.1
Secretin	–	–	–	–	–	–
Glucagon	–	–	–	–	–	–

Summary of the binding parameters (IC_{50}, Hill slope) deduced from experiments of competitive displacement of ^{125}I-VIP, ^{125}I-PACAP 27 or ^{125}I-PHI, by VIP and VIP analogues in chicken liver membranes. The data correspond to binding curves presented in Fig 1A, 1B, 1C.

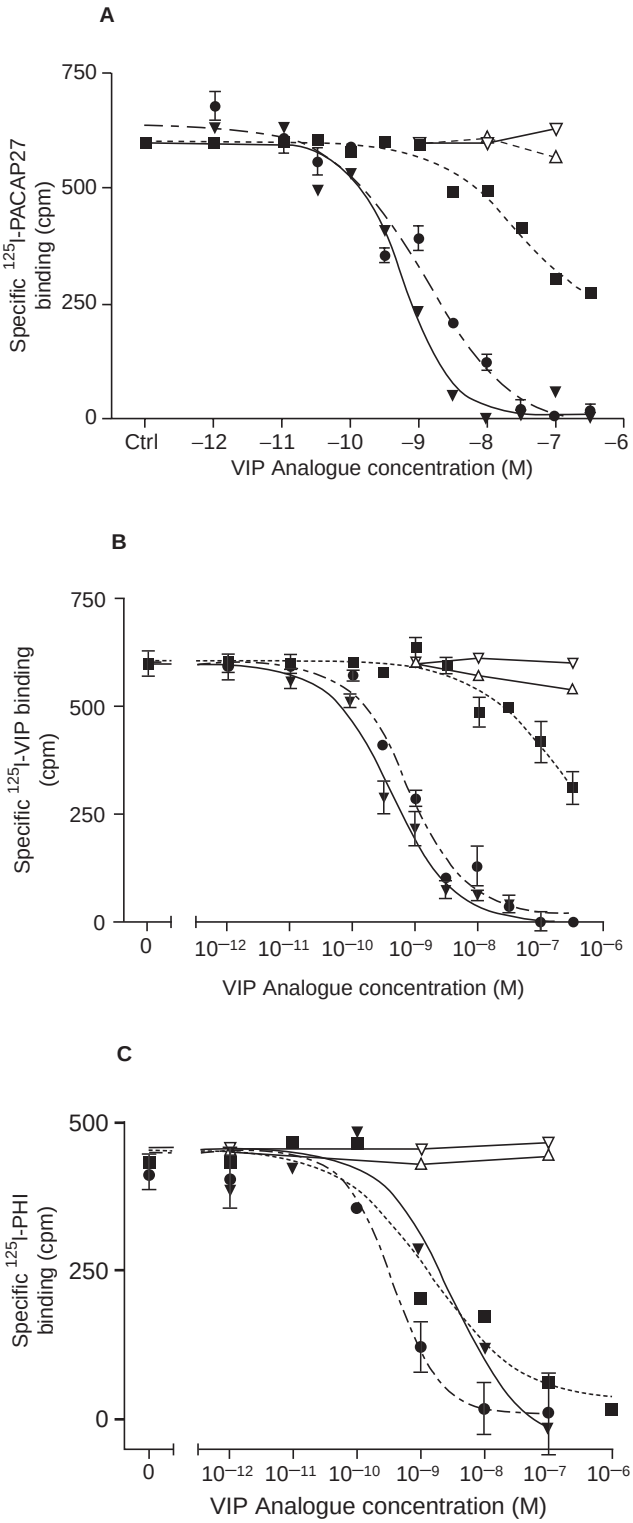

Fig. 1 Competitive displacement of ^{125}I-PACAP 27 (A), ^{125}I-VIP (B) or ^{125}I-PHI (C), by VIP and VIP analogues in chicken liver membranes. Membranes were incubated for 50 min at 20°C in the presence of the radioligand and of the analogues at the indicated concentrations:

GLUCAGON (△), PACAP27 (●), PHI (■), SECRETIN (▽) or VIP (▼). Data represented the mean + /− SEM of the 3 independent experiments, each performed in triplicate.

displayed a much lower affinity for ^{125}I-PACAP27 (33nM) or ^{125}I-VIP (28.6 nM) than for ^{125}I-PHI (0.54nM) binding species. Generally, Hill slope values close to 1 were obtained in all displacements curves where VIP was used as a unlabeled ligand while low Hill slope values of 0.6 to 0.7 were observed with unlabeled PHI.

Furthermore, Kd and Bmax values were also obtained for each type of binding site, after SCATCHARD analysis of the data from displacement curves of each radioiodinated peptide by the corresponding unlabeled peptide; in all cases, Kd values were very similar to the corresponding IC_{50} values obtained by non-linear analysis:

For ^{125}I-PACAP27 displacement by PACAP 27: Bmax = 5 fmol/mg; Kd = 1 nM

For ^{125}I-VIP displacement by VIP: Bmax = 5 fmol/mg; Kd = 0.3 nM

For ^{125}I-PHI displacement by PHI: Bmax = 3,5 fmol/mg; Kd = 0.5 nM

Effects of a non hydrolysable GTP analogue (GPPNHP) on the specific binding of ^{125}I-PHI or ^{125}I-VIP

In order to assess the coupling state of the receptors (coupling to G proteins), the effects of increasing concentrations ranging from 10^{-12}M to 10^{-4} M of a non hydrolysable GTP analogue (GppNHp) were tested on the specific binding of ^{125}I-PHI or ^{125}I-VIP radiotracers (Fig. 2). The data indicated that the action of the nucleotide resulted in an about 50% reduction of the specific ^{125}I-VIP binding and a 30% reduction of the specific ^{125}I-PHI binding, even at the highest concentration utilized of 0.1 mM. The maximal effect of GppNHp was already observed for 20 μM nucleotide while half-maximal reduction of the radioligand binding was obtained for about 10 nM nucleotide.

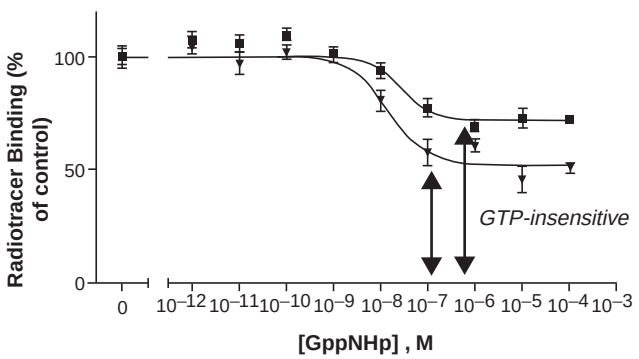

Fig. 2 Dose-dependent inhibition by the GTP analogue, Gpp-NHp, of the ^{125}I-PHI or of the ^{125}I-VIP specific binding, in liver membranes. Membranes (100 μg/test) were incubated at equilibrium (50 min at 20°C) with ^{125}I-VIP (30 pM; ▼) or ^{125}I-PHI (30 pM; ■) and indicated increasing concentrations of nucleotide. Results are expressed as the percentage of the ^{125}I-VIP by radiotracer specifically bound in the absence of nucleotide. Each point is the mean +/− SEM of 3 independent experiments, each performed in triplicate.

Comparison of pharmacological profiles of ^{125}I-PACAP 27, ^{125}I-VIP or ^{125}I-PHI binding sites in the absence or in the presence of GppNHp

Since about 70% of the ^{125}I-PHI and 50% of the ^{125}I-VIP binding sites appeared to be unaffected by the action of GTP, the pharmacological profiles of the GTP insensitive binding sites were analyzed using each of the 3 radiotracers ^{125}I-PACAP 27, ^{125}I-VIP or ^{125}I-PHI in the presence of the GTP analogue (Table 2) and compared with the control profiles obtained in the absence of 20 μM GppNHp (already presented in Table 1). The pharmacological profiles performed in the absence or in the presence of 20 μM GppNHp, were quite similar except a very significant 5.5-fold increase of the relative affinity of PHI for the ^{125}I-PACAP 27 binding sites and of 17-fold for the ^{125}I-VIP binding sites in GTP-treated membranes. For instance, in ^{125}I-VIP binding, experiments, the IC_{50} for PHI that was 28.6 ± 0,39 nM in the absence of GTP analogue (Table 1), decreased to 1,7 ± 0,1 nM in the presence of GppNHp (Table 2). The increased affinity of PHI was also paralleled by an increased Hill slope (from 0,7 in control to 1 in GppNHp-treated membranes). To illustrate the increased relative affinity of PHI for the VIP binding sites in GTP-treated membranes, the corresponding displacement curves obtained in the absence (Fig. 3A) or in the presence (Fig. 3B) of the GTP analogue, are also presented here. Another important observation deduced from these curves was that in the presence of GppNHp (Fig. 3B), the specific ^{125}I-VIP binding was completely abolished by PHI while this analogue reduced by only about 60% the radioligand interaction in the control experiment (Fig. 3A).

Time-course of ^{125}I-PHI binding to liver membranes; effect of GppNHp

Low Hill slope values in binding experiments, like those observed in the present study using ^{125}I-PHI, are generally considered to result from an interaction of the radioligand with more than one set of receptors displaying differential binding properties. In order to further characterize the liver membranes ^{125}I-PHI binding sites, experiments of kinetic association of the radiotracer were undertaken, in

Table 2

	^{125}I-VIP displacement		^{125}I-P27 displacement		^{125}I-PHI displacement	
Ligands	**IC_{50} ± SEM (nM)**	**Hill slope ± SEM**	**IC_{50} ± SEM (nM)**	**Hill slope ± SEM**	**IC_{50} ± SEM (nM)**	**Hill slope ± SEM**
VIP	0.27 ± 0.1	1.00 ± 0.1	1.4 ± 0.13	1.00 ± 0.2	0.42 ± 0.2	1.00 ± 0.2
PACAP-27	1.40 ± 0.1	0.9 ± 0.1	1.30 ± 0.2	0.80 ± 0.2	0.30 ± 0.2	0.90 ± 0.15
PHI	1.70 ± 0.1	1.00 ± 0.2	6 ± 0.1	1 ± 0.2	0.5 ± 0.1	0.8 ± 0.26
Secretin	–	–	–	–	–	–
Glucagon	–	–	–	–	–	–

Summary of the binding parameters (IC_{50}, Hill slope) deduced from experiments of competitive displacement of ^{125}I-VIP, ^{125}I-PACAP 27 or ^{125}I-PHI, by VIP and VIP analogues in chicken liver membranes. Binding conditions were similar to those corresponding to Fig. 1 except that membranes were incubated in the presence of 10^{-5} M GppNHp.

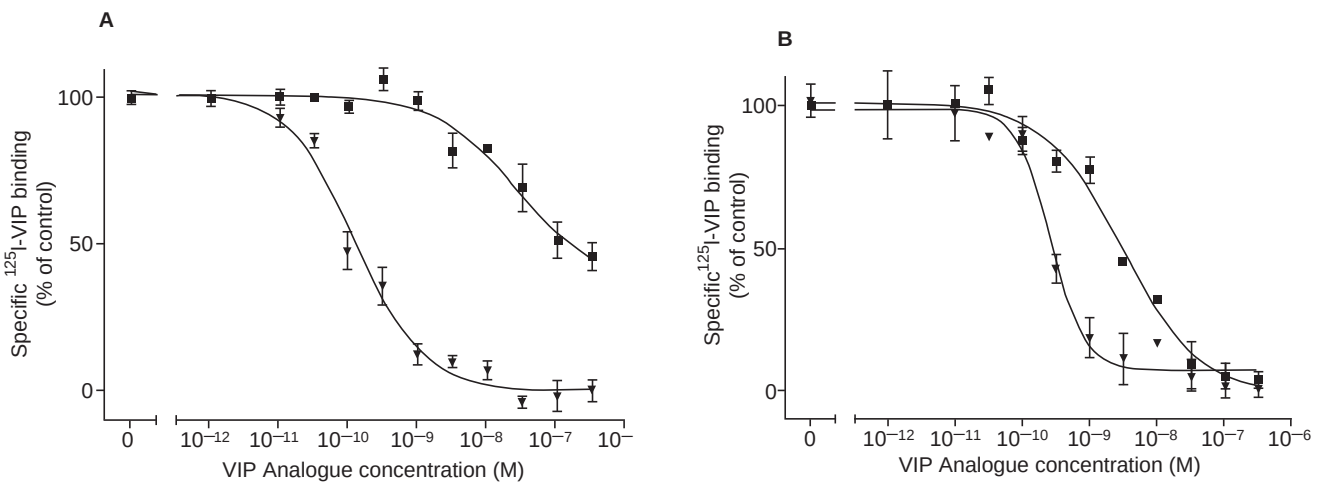

Fig. 3 Competitive inhibition of the specific ^{125}I-VIP binding, in chicken liver membrane by VIP (▼) or PHI (■), in the absence (A) or in the presence (B) of 20 μM of GppNHp. Membranes were incubated for 50 min at 20°C in the presence of the radioligand and of the specified analogs at the indicated concentrations. Data represent the mean +/− SEM of 3 independent experiments, each performed in triplicate.

the absence or in the presence of the GTP analogue. Under our experimental conditions, equilibrium was reached after 60 min at 20°C (Fig. 4A). In the absence of GTP analogue, the kinetics of association revealed two components which were clearly evidenced after linearization of the data (Fig. 4B). The apparent association constants (Kobs) for these 2 sites, obtained after computer assisted analysis of the data were $0.056 \pm 0.0051 \text{min}^{-1}$ and $0.0138 \pm 0.0004 \text{ min}^{-1}$. In the presence of 20 μM GppNHp, the kinetics of association revealed one single component having an apparent association constant (Kobs) of $0.057 \pm 0.002 \text{ min}^{-1}$.

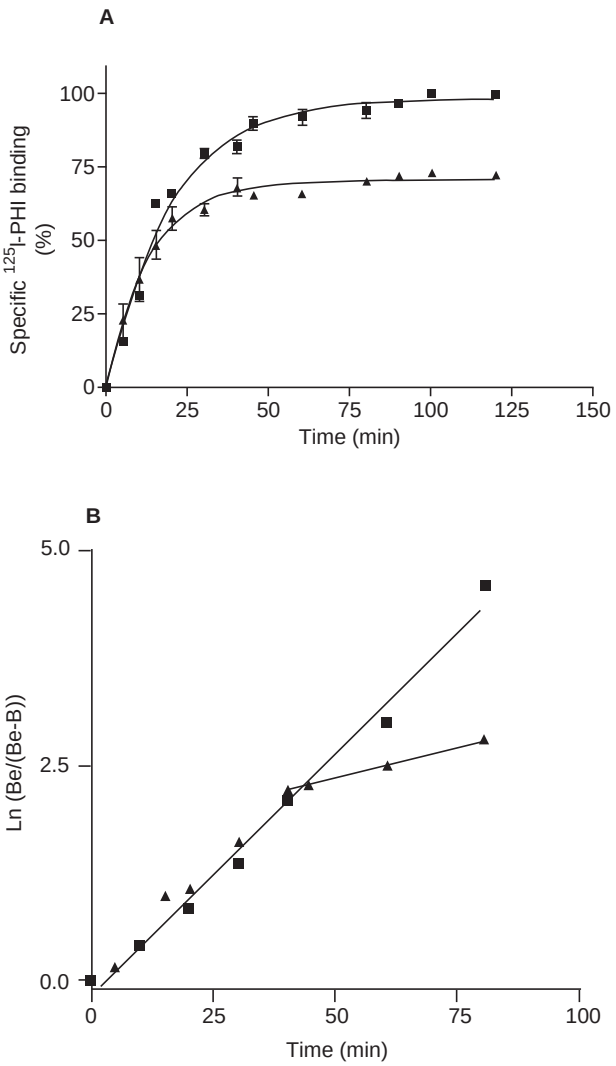

Fig. 4 Kinetic association binding of ^{125}I-PHI in liver membranes in the absence or in the presence of GppNHp. Membranes were incubated for the indicated times at 20°C in the presence of ^{125}I-PHI, in the absence (■) or in the presence (▲) of ≒ 20μM GppNHp (Fig. 4A). Data represented the mean +/− SEM of 3 independent experiments, each performed in triplicate. Data from the same experiments were linearized, in order to assess the Kobs values for each association kinetics (Fig. 4B). B: specific binding at a given time of incubation, Be: specific binding at equilibrium, Kobs = association constant.

Covalent cross-linking of ^{125}I-VIP to liver membrane proteins

The relative molecular mass of the VIP binding sites was approached by covalent cross-linking of ^{125}I-VIP, using the bifunctional reagent BSOCOES. As presented in Fig. 5, in the control experiment, one major component of Mr = 60,000 (pointed by arrow B) and a second receptor of Mr = 48,000 (pointed by arrow A) were resolved, using SDS-PAGE and autoradiography. The radiolabelling of these two different forms was progressively abolished by increasing concentrations (10^{-12} M, 10^{-9} M or 10^{-7} M) unlabled VIP.

Effects on the VIP receptor covalent radiolabelling of increasing concentrations of GppNHp (10^{-12} M, 10^{-8} M and 10^{-5} M were also studied. The data indicate that these treatments selectively and dose-dependently affected the radiolabelling of the Mr = 60,000 component B, which was almost completely abolished, at the highest GppNHp concentration of 10^{-5} M. On the opposite, the radiolabelling of the Mr = 48,000 species was not significantly affected, even at the highest concentration of 10^{-5} M GTP analogue.

The action of increasing concentrations of PHI (10^{-12} M, 10^{-9} M and 10^{-7} M) resulted in a selective dose-dependent inhibition of the radiolabelling of the Mr = 48,000 species which was completely abolished by 10^{-7} M PHM. On the opposite, in our experimental conditions, PHI only partially affected the Mr = 60,000 form radiolabelling, with a maximal inhibition of about 50% at the highest concentration of 10^{-7} M.

Covalent cross-linking of ^{125}I-PHI to liver membrane proteins

The relative molecular mass of the PHI binding sites was approached by covalent cross-linking of ^{125}I-PHI, using the bifunctional reagent BSOCOES. As presented in Fig. 6, in the control experiments, two components displaying a similar labelling intensity, of Mr = 48,000 (pointed by arrow A) and of Mr = 60,000 (pointed by arrow B) were resolved, using SDS-PAGE and autoradiography. However, the labelling of these components by ^{125}I-PHI was less intense than that observed for ^{125}I-VIP. The radiolabelling of the Mr = 60,000 component B was completely and selectively abolished, by a concentration of 10^{-5} M GppNHp, which had no significant effect on the staining of the Mr = 48,000 component A. Conversely, increasing concentrations of unlabeled PHI (10^{-12} M, 10^{-9} M or 10^{-7} M) selectively abolished the ^{125}I-PHI labelling of the

Mr = 48,000 while they were not significantly affecting the labelling of the Mr = 60,000 form B.

DISCUSSION

In a first set of experiments, the relative representation of PACAP, VIP and PHI binding sites in liver membranes was determined, indicating that in this tissue, ^{125}I-VIP and ^{125}I-PACAP binding capacities (Bmax) were about equivalent, hence corresponding most probably to bivalent VIP/PACAP receptors, while the ^{125}I-PHI binding sites were less represented. Globally, the pharmacological profiles also corresponded to the definition of polyvalent high-affinity VIP/PACAP receptors, with very similar IC_{50} values for VIP and PACAP-27 in the nanomolar range and poor or no interaction with secretin or glucagon. High affinity interaction of PHI was only seen in binding experiments where ^{125}I-PHI was used as a radiotracer while PHI interacted with a quite lower affinity with ^{125}I-PACAP (IC50 = 33nM) or ^{125}I-VIP (IC_{50} = 28.6 nM) binding sites. Generally, Hill values obtained in experiments of displacement of ^{125}I-PACAP, ^{125}I-VIP or ^{125}I-PHI by unlabeled PHI were quite low (0.6 to 0.7), indicating that PHI may interact with different subsets of ^{125}I-PACAP, ^{125}I-VIP or ^{125}I-PHI binding sites displaying differential PHI binding affinities. On the opposite, Hill values obtained in experiments of displacement of ^{125}I-PACAP, ^{125}I-VIP or ^{125}I-PHI by unlabeled VIP were closer to 1, suggesting that unlabeled VIP was not discriminating as efficiently as PHI these potential binding subsites. To further characterize these liver subsites, a non hydrolysable GTP analogue, GppNHp was utilized to discriminate the coupling state of these receptors. About 50% of the ^{125}I-VIP receptors and 70% of the ^{125}I-PHI binding species appeared to be GTP-insensitive. This led us to determine the pharmacological profiles of these GTP-insensitive binding sites for the 3 radiotracers considered in this study. Globally, the pharmacological profiles obtained in the presence of the GTP analogue resembled

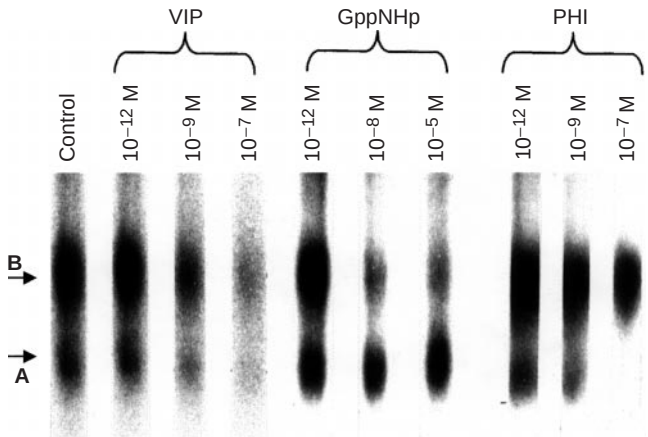

Fig. 5 Specific covalent labelling of chicken liver membrane proteins by ^{125}I-VIP detected by SDS-PAGE and autoradiography. Membranes (200μg) were incubated with ^{125}I-VIP (for 50 min at 20°C) in the absence or in the presence of the specified additions, then treated with 2 mM cross-linked reagent BSOCOES. The two radiolabelled components called A (Mr = 48,000) and B (Mr = 60,000) are indicated by arrows.

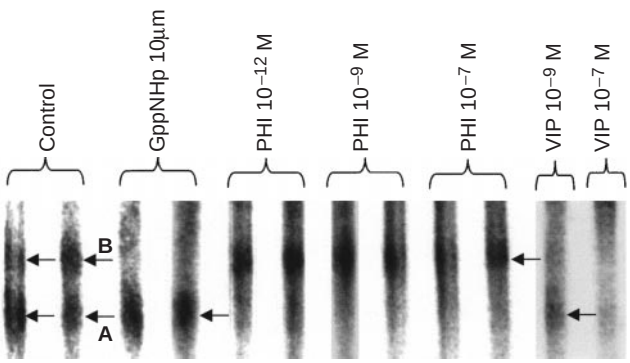

Fig. 6 Specific covalent labelling of chicken liver membrane proteins by ^{125}I-PHI detected by SDS-PAGE and autoradiography. Membranes (200μg) were incubated with ^{125}I-PHI (for 50 min at 20°C) in the absence of in the presence of the specified additions, then treated with 2 mM cross-linked reagent BSOCOES. The two radiolabelled components called A (Mr = 48,000) and B (Mr = 60,000) are indicated by arrows.

those observed for control experiments conducted in the absence of GppNHp. However, at least two important observations indicated a different pharmacological behaviour of the binding sites in GppNHp-treated membranes, when compared with control experiments:

1. PHI competed only with about half of the ^{125}I-VIP binding sites in control experiments while a complete inhibition of ^{125}I-VIP binding by PHI was observed in GppNHp incubated membranes.
2. a spectacular increase of the relative affinity of PHI for the ^{125}I-PACAP/^{125}I-VIP binding sites occurred, following membranes treatment with 20 μM GppNHp.

Taken together, these pharmacological data indicated that the fraction of the ^{125}I-VIP binding sites (about 50%) of control liver membranes that did not interact with PHI, could correspond to the fraction of the ^{125}I-VIP receptors that were sensitive to the GTP analogue. In addition, PHI appeared like an high-affinity ligand for the fraction of the ^{125}I-VIP binding species that was insensitive to the GTP analogue. It thus appeared attractive to analyse further whether ^{125}I-PHI could discriminate a GTP-insensitive state of the polyvalent VIP/PACAP receptors. This was checked in experiments of kinetic association of ^{125}I-PHI, in the presence of in the absence of the GTP analogue. The data confirmed that ^{125}I-PHI interacted with 2 classes of binding sites in control experiments, the higher affinity site displaying an about 4-fold higher association constant (Kobs) than that of the lower affinity site. Treatment of the membranes with the GTP analogue converted these 2 sites into one single binding species with a similar Kobs than that of the high affinity binding site obtained in control experiments.

The molecular nature of the liver VIP and PHI receptors represented in liver membranes was then approached trough experiments of covalent cross-linking of ^{125}I-VIP or ^{125}I-PHI. Two distinct receptors of Mr = 48,000 and Mr = 60,000 were identified, using this approach. In summary:

- Both species were more efficiently radiolabelled by ^{125}I-VIP than by ^{125}I-PHI, which could account for the lower binding capacity observed in this tissue for radioiodinated PHI than for radioiodinated VIP. The Mr = 60,000 component was the major radiolabelled species, using ^{125}I-VIP, while ^{125}I-PHI autoradiographic density was about equivalent for both species.
- Both species corresponded to high affinity VIP receptors, since unlabeled VIP abolished the radiolabelling of both forms in a similar manner, as well in experiments using ^{125}I-VIP or ^{125}I-PHI.
- The 60 kDa species was highly sensitive to the GTP analogue : GppNHp strongly inhibited the 60 kDa form radiolabelling by ^{125}I-VIP and completely abolished its radioactive staining by ^{125}I-PHI. On the opposite, no significant effect of the GTP analogue on the radiolabelling of the 48 kDa species could be observed, as well in experiments using ^{125}I-VIP as in those using ^{125}I-PHI as a radiotracer.
- The 48 kDa species was discriminated by unlabeled PHI : using both radioligands, the labelling of the 48 kDa species was selectively, dose-dependently and strongly affected by unlabeled PHI while the labelling of the 60 kDa component was only very partially inhibited.

Data from different groups conducted to quite disparate results concerning the molecular mass of the cross-linked VIP receptor, probably due to some variability in the experimental conditions. To summarize, species ranging from 48 to 55 kDa were identified in pig and rat tissue together with higher species of 77–86 kDa in rat. The human VIP receptor was characterized as a 61 kDa component (Favre et al., 1993). A Mr = 150,000 solubilized complex containing a VIP receptor and the β-subunit of the Gs complex has also been reported in the rat liver(Couvineau et al., 1990).

The cross-linking data reported here closely correlate those previously reported by other investigators, demonstrating a GTP-insensitive Mr = 46,000 VIP receptor expressed in rat brain, coexisting with a Mr = 64,000 GTP-sensitive component (Gourlet et al., 1998). Furthermore, we propose that these two forms of VIP receptors can be discriminated through their differential interaction with the VIP analogue PHI which appears like an high-affinity selective ligand for the 48 kDa GTP-insensitive component.

The properties of the liver GTP-insensitive VIP/PHI receptor are to be put together with previous reports on binding of ^{125}I-PHI in rat liver, describing two classes of binding sites in this tissue : a PHI-preferring species which interacted poorly with VIP, and a second site which was equally reactive with PHI and VIP (Paul et al., 1987); the latter could correspond to the Mr = 48,000 VIP/PHI receptor described in the present study.

Two research groups independently reported the stable expression of the recombinant VIP1 receptor in clonal CHO cells (Gaudin et al., 1996; Ciccarelli et al., 1994). The overexpressed receptor corresponded to a Mr = 70,000 form (Gaudin et al., 1996). The pharmacological profile of the recombinant receptor indicated that this species interacted with PHI with a much lower affinity than VIP. Such low affinity for PHI is also a property of the Mr = 60,000 form characterized in our study.

We have concurrently characterized the Mr = 48,000 species in the chicken and rat brain brain and in human T-lymphoblastoma cells and confirmed the high affinity of this receptor for PHI, hence demonstrating that this 48 kDa species is not limited to the liver (data not shown).

In conclusion, we propose that based on their differential sensitivities to GTP and PHI, two polyvalent VIP/PACAP molecular forms of VIP binding sites can be distinguished in the chicken liver: the first is a GTP-insensitive, Mr = 48,000 receptor that shares similar high affinity for VIP, PACAP and PHI. The second is a Mr = 60,000 GTP-sensitive receptor that interacts poorly with PHI. The latter could correspond to the well-characterized VIP1 receptor subtype whose corresponding cDNA has been cloned years ago from a human liver library (Gagnon et al., 1994).

It was long established in early reports on ^{125}I-VIP binding studies that only part of the VIP binding sites expressed in the liver were sensitive to GTP (Hill et al., 1992; Amiranoff et al., 1980). More recent data led to the proposal that GTP-insensitive receptors may trigger growth and trophic properties of VIP. These 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 CNS and of the somites (Gressens et al., 1993). In liver, such GTP-insensitive receptors involved in growth and trophic regulations could also take part in important biological and physiological processes, such as hepatic regeneration. In a recent article from the group of Hill, it was reported that a VIP derivative (stearyl-norleucine 17 VIP) could discriminate a subset of GTP-insensitive VIP binding sites in the CNS (Hill et al, 1999). We propose here the prominent statement that these receptors can also be pharmacologically discriminated by the VIP analogue PHI and that they could correspond to a distinct 48 kDa molecular species. Investigation on the molecular nature and on the function of this Mr = 48,000 GTP-insensitive species as well as on the associated intracellular transduction mechanisms, thus appears as a priority for future prospects.

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