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Identification of a Pituitary Growth Hormone-Releasing Peptide (GHRP) Receptor Subtype by Photoaffinity Labeling

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ABSTRACT
Hexarelin, an analogue of GHRP-6, in which D-Tryptophan has been replaced by its 2-methyl derivative, is known to release growth hormone (GH) in vivo and in vitro by direct action on receptors present in anterior pituitary cells. Measurement of second messengers (c-AMP, Ca^{++}, IP_3) upon somatotrophs stimulation, suggests the existence of more than one GHRP receptor subtype. In order to document such an hypothesis, we have used a new photoactivatable derivative of Hexarelin, Tyr-Bpa-Ala-Hexarelin. This derivative was shown to be fully active in the release of GH in vivo with neonate rats. Using this photoactivatable ligand, we have specifically labeled a protein with an apparent M_r of 57 000 in human, bovine and porcine anterior pituitary membranes. Hexarelin and the spiroindoline sulfonamide MK-0677 displaced the M_r -57 000 photolabeled band with an apparent ED_{50} of $6x10^{-7}$ M and $2x10^{-5}$ M respectively. Taking into account the high efficiency (>60%) of covalent incorporation of the Bpa residue, this photoactivatable Hexarelin derivative has allowed the identification of a pituitary GHRP receptor subtype, which is apparently distinct from the recently cloned GH secretagogue receptor.

GROWTH hormone-releasing peptides (GHRPs) modeled from Met-enkephalin have been developed by combining conformational energy calculations, peptide chemistry and structure-activity relationship (1-4). Among various synthesized peptides, GHRP-6 (His-DTrp-Ala-Trp-DPhe-Lys-NH_2) behaves as a potent GH secretagogue (GHS) in vivo and in vitro (4), and displays low affinity binding to opioid receptors (5). In vitro GHRP-6 is able to stimulate the secretion of GH in isolated bovine and rat anterior pituitary cells (6,7). Moreover, binding studies with pituitary and hypothalamus membranes show saturability, specificity and reversibility (8-10). Hexarelin (His-D2MeTrp-Ala-Trp-DPhe-Lys-NH_2) has been described as being less susceptible to degradation than GHRP-6 (11) and its administration to humans stimulates GH release in a dose dependent fashion with a high potency (12). Recently, a new class of non-peptidic GH-releasing compounds has been developed. The spiroindoline MK-0677 (13) and the benzolactam L-692,429 (14) have been reported to stimulate GH secretion in vivo and in vitro. High affinity [^{35} S] MK-0677 binding to receptors present in porcine pituitary membranes has been found to be inhibited by GTP-gamma-S and to be displaced by Hexarelin and GHRP-6 (15).

Recently, a GHS receptor has been cloned from porcine, human (16) and rat (17) pituitary libraries. Two different cDNAs encoding for a 366 amino acids protein (type Ia) and a 289 amino acids protein (type Ib) were described (16). Binding studies done with membranes from COS-7 cells expressing human type Ia cDNA, show the pharmacological pattern that is reported for pituitary membranes (15). On the other hand the protein encoded by type Ib cDNA is devoid of binding activity. Comparison of human and porcine type Ia receptor sequences reveals 93 % identity. This receptor features seven predicted transmembrane domains, three potential sites for N-linked glycosylation and ten conserved cysteine residues.

In order to identify the presence of GHRP receptor subtypes in different tissues or species, we have developed a photoreactive derivative of Hexarelin, using the photoactivatable amino acid derivative *p*-benzoyl-L-phenylalanine (Bpa). We report, herein, the identification of a pituitary GHRP receptor subtype by photoaffinity labeling using [^{125}I]iodoTyr-Bpa-Ala-Hexarelin as photoprobe.

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Materials and Methods

Chemicals

Hexarelin and MK-0677 were provided by Europeptides (Argenteuil, France). Standard Fmoc amino-acids were from I.A.F. Biochem (Laval,Canada) and Bachem (Bubendorf, Switzerland). Endoglycosidase F/N-Glycosidase F, was from Boehringer Mannheim (Mannheim, Germany). Carrier free $Na^{125}I$ was from Amersham Corp. (Oakville, Canada). Electrophoresis molecular weight markers were from Pharmacia (Uppsala, Sweden). All other reagents were from commercial sources.

Peptide Synthesis.

The peptide Tyr-Bpa-Ala-His-D2MeTrp-Ala-Trp-DPhe-Lys-NH_2 was obtained by solid-phase synthesis and purified on a preparative reverse-phase (C18) column (2.5 cm x 30 cm) with a linear gradient of acetonitrile in 0.05% trifluoroacetic acid (TFA). Mass number of the photoactivatable Hexarelin derivative, assessed by Fast Atom Bombardment Mass Spectrometry (M^+ = 1372), was in accordance with its expected molecular weight (Mw = 1371.56).

Membrane Preparation

Frozen human, bovine and porcine anterior pituitaries were homogenized at 4 C in 50 mM Tris-HCl, pH 7.4, 2 mM EGTA, 0.1 mM Pefabloc, 1 μM leupeptin, 1 μM aprotinin, and 1 μM pepstatin A. The homogenate was centrifuged at 500 x g for 10 min and the supernatant was centrifuged at 20 000 x g for 20 min. The resulting pellet was washed twice, resuspended in the

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homogenization buffer and frozen in liquid nitrogen. Gx cells (a cell line derived from a mammosomatotroph adenoma) (18) were washed and homogenized in 50 mM Tris-HCl, pH 7.4, containing 5 mM $MgCl_2$, 5 mM EDTA, and 250 mM sucrose. After a low spin at 1000 x g for 10 min, the supernatant was centrifuged at 67 000 x g for 20 min. The resulting pellet was frozen at -80 C. Protein concentration was determined with the bicinchoninic acid method, using BSA as standard.

Iodination of Bpa-Hexarelin.

The iodination procedure was performed in the darkness. Tyr-Bpa-Ala-Hexarelin (10 nmol) was mixed with 100 ng of lactoperoxidase and 1 mCi of $Na^{125}I$ in a volume of 40 µl of 0.1N sodium acetate buffer pH 5.6. The reaction was started by adding 5 µl (3 nmol) of H_2O_2. The incubation was carried out at 22 C for 5 min. The addition of H_2O_2 was repeated twice with 5 min incubation each time. The iodinated peptide was purified on a reverse-phase Vydac C_{18} column with a 60 min linear gradient (1 ml/min) from 18% to 45% acetonitrile in 0.1% TFA.

Receptor Binding and Photolabeling with[^{125}I]iodo Bpa-Hexarelin.

Membranes were incubated in the darkness, in 50mM Tris-HCl pH 7.4 , 2mM EGTA , 0.1% Bacitracin (Buffer A) in the presence of the indicated concentration of [^{125}I]iodoBpa-Hexarelin. After an incubation period of 60 min at 22 C, membranes were submitted to irradiation with UV lamps (365 nm) for 15 min at 4 C. After centrifugation at 12 000 x g for 10 minutes, the pellets were resuspended in sample buffer (62 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 15% 2-mercapto-ethanol, and 0.05% bromophenol blue), and boiled for 5 min prior to be subjected to 7.5% SDS-PAGE (19). Autoradiographic exposure was done at -80 C using Dupont film with a reflection intensifying screen. Buffer A was degassed under vacuum, sparged with nitrogen, and used in capped tubes in order to minimize lipid peroxydation.

Deglycosylation

The photolabeled receptor was purified on a 3mm thick 7.5% SDS-PAGE (19). Bands corresponding to a molecular weight of 57 000 were cut in 1mm^3 slices and eluted 2 times with 5 volumes of 0.01% SDS in 50 mM NH_4HCO_3. Proteins were concentrated on Centricon-30 (Millipore) to 2 mg/ml. Deglycosylation was performed with N-Glycosidase F at 50mU/µg of protein in 250 mM sodium phosphate buffer pH 7.5, 10mM mercaptoethanol, 10 mM EDTA, and 0.6% Octyl Glucoside for 24 hours at 25 C.

Results

Synthesis and biological activities of a photoactivatable Hexarelin derivative.

Prior knowledge on the structure-activity relationship of GHRPs is required before modifying Hexarelin by inserting a tyrosine and a Bpa residue for iodination and photolabeling respectively. Amidation and the presence of an aromatic amino acid residue at position 2 (1,2) appear to be essential for GH-releasing activity of the GHRPs analogs. The L-D-X-L-D sequence of the pentapeptide (Tyr-DTrp-Ala-Trp-DPhe-NH_2) is crucial for a correct spatial orientation of the ring sets which are essential for the GH-releasing activity. The presence of histidine at position 1, and the extension at the carboxy-terminus with the positively charged amino-acid lysine gives a more potent GH-releasing peptide (4). Based on the model obtained by low energy conformation of its parent peptide, we assumed that modification of Hexarelin was possible only at its N-terminus. Therefore, tyrosine was added at the amino end, spaced by an alanine in order to distance it from the core of the peptide, to obtain Tyr-Ala-Hexarelin (Tyr-Ala-His-D2MeTrp-Ala-Trp-DPhe-Lys-NH_2).This intermediate analogue conserved the full agonist activity of the original GHRP. Figure 1 shows that Tyr-Ala-Hexarelin stimulates the rat GH secretion by 6.8 fold over the control ($p<0.01$), reaching a level comparable to that obtained in rats injected with Hexarelin. Positioning the tyrosine at the extremity of the peptide also favours its radioiodination in a mild oxidative condition which minimizes the formation of diiodinated peptide (data not shown).

In order to increase the specificity of the photolabeling, the Bpa residue was localized between the tyrosine and the alanine. We assumed that its positionning close to the bioactive region of the peptide allowed it to point towards the binding site of the receptor. The synthesized Bpa-Hexarelin (Tyr-Bpa-Ala-His-D2MeTrp-Ala-Trp-DPhe-Lys-NH_2) behaves as a full agonist inducing a significant increase of rat GH plasma level ($p<0.01$) (Fig. 1). Iodination of Bpa-Hexarelin yielded a radioligand with a specific activity of 2000 Ci/mmol. The [^{125}I]monoiodo Bpa-Hexarelin eluted at 37% of acetonitrile in 0.1% TFA on a C_{18} reverse-phase column (data not shown).

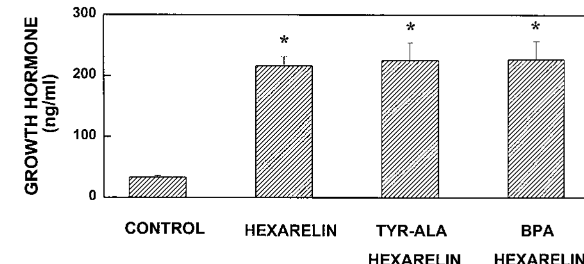

Fig 1: Plasma GH response to s.c. administration of 300 µg / Kg of various peptides in 10 days-old rats. GH was measured 15 min after the injection, by RIA using a double antibody method according to Locatelli et al. (20).
* $P<0.01$ vs saline-injected controls (analysed by one way ANOVA and Dunnett's *t* test).

Covalent photolabeling with[^{125}I]iodoBpa-Hexarelin.

The radiolabeled peptide was assayed for its capability to bind irreversibly to its receptor, using pituitary membrane preparations obtained from different species and from the Gx cell line (Fig.2A). Photolabeling of porcine and bovine anterior pituitary membranes shows a major band with an apparent molecular weight of 57 000. This band is fully displaced in the presence of 10^{-5} M Hexarelin. With human pituitary membranes, a doublet is visible at M_r of 53 000 and 57 000, but only the M_r 57 000 band is displaced by Hexarelin (Fig. 2A). With the Gx cell line, a specifically photolabeled band of M_r 57 000 is also visible but of lower intensity. Increasing the time of irradiation failed to specifically photolabel any other band. The radical scavenger *p*-aminobenzoic acid used at 10^{-4} M did not affect the electrophoretic pattern of the photolabeled preparation (data not shown). Staining the proteins with Coomassie blue demonstrated that there was no major protein in the region

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corresponding to molecular weight of 57 000. When the electrophoresis was carried out under non-reducing conditions, the major M_r 57 000 band was still apparent without any higher molecular weight band (Fig. 2B). These results indicate that the photolabeled GHRP receptor consists of a single polypeptidic chain of M_r 57 000. Since the calculated molecular weight of the cloned receptor is about 41 000 (16), the difference in size with the photolabeled protein could be explained by glycosylation of the molecule. However, Fig. 2C shows only a slight change in protein size (M_r 54 000 vs M_r 57 000) after treatment with n-Glycanase, indicating its low degree of glycosylation at least in bovine pituitaries. Since the structure of Hexarelin is modeled from that of enkephalins, we assessed the effect of opioids on the binding of [^{125}I]iodoBpa-Hexarelin. As shown in Fig. 2D, Leu-enkephalin at 10^{-5} M was unable to suppress the photolabeling of the M_r 57 000 band.

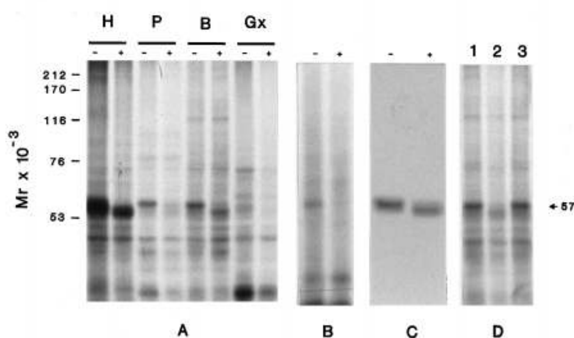

Fig 2: A) Covalent photolabeling of the GHRP receptor with [^{125}I]iodoBpa-Hexarelin in membranes obtained from human (H), porcine (P) or bovine (B) pituitaries and the Gx cell line. Membranes (400 µg of protein) were incubated with 10^6 cpm of [^{125}I]iodoBpa-Hexarelin with (+) or without (-) 10 µM of Hexarelin for 60 min at 22 C. Tubes were then exposed to UV light, centrifuged and resuspended in sample buffer. Lanes were loaded with 200 µg of protein and subjected to SDS-PAGE. B) Bovine pituitary membranes were treated as above but the electrophoresis was run in the absence of 2-mercaptoethanol. C) SDS-PAGE of photolabeled bovine pituitary membranes treated (+) or not treated (-) with n-Glycanase. D) Photolabeling of bovine pituitary membranes in the absence (lane 1) or in the presence of 10 µM of Hexarelin (lane 2) or 10 µM of Leu-enkephalin (lane 3).

In order to evaluate the affinity and the specificity of the covalent attachment of [^{125}I]iodoBpa-Hexarelin to the M_r 57 000 protein, photolabeling was performed using bovine pituitary membranes and saturating concentrations of the radioligand in the presence and in the absence of Hexarelin. After autoradiography, the gel was placed in a PhosphorImager in order to quantify the intensity of the bands. Figure 3 shows the progressive saturation of the specific M_r 57 000 band and the PhosphorImager intensity curves, whose integration resulted in a half-saturation concentration of 2.8×10^{-8} M. Figure 4 shows the Hexarelin and MK-0677 competition with the photolabeling agent [^{125}I]iodoBpa-Hexarelin, for the GHRP receptor in bovine pituitary membranes. Hexarelin and MK-0677 compete for the M_r 57 000 band with ED_{50} of 0.6 µM and 20 µM respectively.

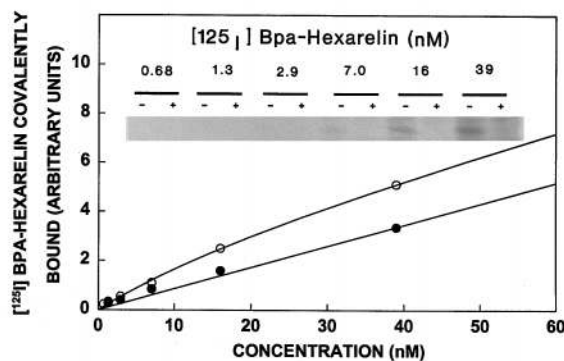

Fig 3: Covalent saturation of the GHRP receptor in bovine anterior pituitary membranes with increasing amounts of [^{125}I]iodoBpa-Hexarelin (quenched to 20 Ci/mmol). Membranes (400 µg) were incubated with (+) or without (-) 10 µM of Hexarelin. Tubes were irradiated and 200 µg of protein were loaded on SDS-PAGE and revealed by autoradiography (inset). Signals detected at M_r 57 000 were quantified by PhosphorImager (Molecular Dynamics) and the result was analysed using ALLFIT for Windows.(O) incubation without Hexarelin, (●) incubation with Hexarelin 10^{-5} M.

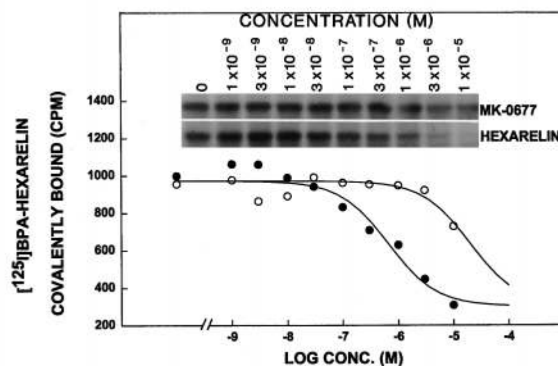

Fig 4: Hexarelin and MK-0677 competition with BPA-Hexarelin. [^{125}I]iodoBpa-Hexarelin was incubated with bovine anterior pituitary membranes (400 µg) in the presence of increasing concentrations (10^{-9} to 10^{-5} M) of Hexarelin or MK-0677. Following UV irradiation, 200 µg of protein were loaded on SDS-PAGE. The incorporated radioligand was visualised by autoradiography (inset). The corresponding M_r 57 000 bands were cut out and the radioactivity measured in a γ counter. The resulting competition curves were evaluated by least-squares, non-linear regression analysis using the software ALLFIT for Windows. (●) Hexarelin and (O) MK-0677.

Discussion

Several lines of evidence indicate that GHRPs exert their action via distinct signaling pathways and through a growing family of receptor subtypes. Until now, attempts to characterize the receptor subtypes using equilibrium binding methods have been unsuccessful. Furthermore, binding studies performed with peptidic GHRPs (8-10) have revealed the presence of binding sites in the pituitary and hypothalamus which characteristics are not correlated with the powerful secretagogue activity of the peptides. This may be due to the high hydrophobicity of the GHRPs and their loss in the phospholipid membrane during the binding assay.

In this paper we have shown that the radiolabeled Bpa-Hexarelin is capable of specifically photolabeling a protein of M_r 57 000 in human, bovine and porcine pituitary membranes and, to a

lesser extent, in membranes obtained from the human mammosomatotroph cell line Gx. The choice of a Bpa derivative instead of chemical cross-linking agents or azido derivatives was based on its high efficiency of covalent photolabeling (21). The introduction of tyrosine and *p*-benzoyl-phenylalanine at the N-terminus of Hexarelin, using alanine as spacer, did not alter the biological activity of this new compound.

Covalent saturation of the M_r 57 000 band with [^{125}I]iodoBpa-Hexarelin showed a half-saturation concentration of 2.8×10^{-8} M comparable to the value reported for the high affinity binding site in rat pituitaries (8,9). In porcine pituitary membranes, Veeraragavan (10) reported a single class of binding sites for GHRP-6 sharing the same affinity (Kd $\sim 10^{-5}$ M) as the low affinity binding site observed in rat pituitary membranes (8,9). However, we have observed also the presence of the specifically labeled M_r 57 000 protein in porcine pituitary membranes. It could be possible that the electrophoresis step unveils the specific binding otherwise masked by a high non-specific binding.

Binding studies in porcine pituitary membranes using the ^{35}S-labeled MK-0677, showed that both Hexarelin and MK-0677 share the same Ki of 0.3×10^{-9} M (15). Surprisingly, when we tested the displacement potency of these GH secretagogues on the M_r 57 000 protein, MK-0677 behaved as a weak competitor compared to Hexarelin. This discrepancy can be explained by the labeling by Bpa-Hexarelin of a protein different from that identified with the non-peptidic compound, suggesting the existence of GHRP receptor subtypes. The existence of receptor subtypes in the ovine pituitaries showing differences in response to various secretagogues have already been postulated (22). Moreover, deglycosylation of the M_r 57 000 protein failed to reduce its size to the calculated molecular weight (~ 41 000) of the protein encoded by type Ia cDNA (16). Taken together these results suggest that Bpa-Hexarelin derivative is able to photolabel a protein which could be analogous to the binding sites reported by Codd (8) and by Sethumadhavan (9), and different from the protein encoded by type Ia cDNA (16). A decade after the discovery of GHRPs as GH secretagogues, this is the first report on the use of a photoactivatable peptidic derivative of GHRP in the identification of a pituitary GHRP receptor subtype.

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