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Binding sites for growth hormone-releasing peptide

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Summary Growth hormone-releasing peptides (GHRPs) are known to release growth hormone (GH) *in vivo* and *in vitro* by a direct action on receptors in anterior pituitary cells. Measurement of second messengers released following somatotroph stimulation suggests the existence of more than one GHRP receptor subtype in the hypothalamic–pituitary system. Furthermore, hexarelin, a hexapeptide of the GHRP family and a potent GH secretagogue, is reported to increase left ventricular ejection fraction, suggesting the expression of specific myocardial GHRP binding sites. In order to confirm such a hypothesis, a photoactivatable derivative of hexarelin, Tyr-p-benzoyl phenylalanine-Ala-hexarelin, was developed. A putative GHRP receptor with an apparent relative molecular mass of 57 000 was specifically labelled and characterized in human, bovine and porcine anterior pituitary membranes using this hexarelin derivative. The existence of myocardial binding sites was also demonstrated using the same approach. The differential binding affinity of GHRP analogues to cardiac tissue raises the possibility of the existence of distinct GHRP receptor subtypes in the pituitary and the cardiovascular system, for which physiological roles have yet to be determined.

Key words: GHRP, hexarelin, pituitary GHRP binding sites, cardiac GHRP binding sites, receptor subtypes.

INTRODUCTION

It is now well established that growth hormone-releasing peptides (GHRPs) and nonpeptidyl growth hormone (GH)-releasing compounds, such as the spiroindoline derivative MK-0677, behave as potent GH secretagogues *in vivo* and *in vitro*,[1,2] acting via a different pathway to that of GH-releasing factor. MK-0677, which binds with high affinity to receptors present in porcine pituitary membranes,[3,4] has been used to characterize the GH secretagogue receptor that has recently been cloned from porcine, human and rat pituitary libraries.[4,5] Hexarelin, a 2-methyl derivative of GHRP-6, has been reported to stimulate GH release in a dose-dependent manner in humans.[6] It has also been observed that hexarelin increases left ventricular ejection fraction in normal individuals,[7] raising the possibility of specific myocardial GHRP binding sites.

In order to detect GHRP receptor subtypes in different tissues, a photoreactive derivative of hexarelin was developed, incorporating a photoactivatable amino acid derivative, p-benzoyl phenylalanine (Bpa).

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MATERIALS AND METHODS

Peptide synthesis

The peptide Tyr-Bpa-Ala-His-D-2-MeTrp-Ala-Trp-D-Phe-Lys-NH_2 was obtained by solid-phase synthesis, purified on a preparative reverse-phase (C18) column with a gradient of acetonitrile in trifluoroacetic acid, 0.05%, and identified by fast-atom bombardment mass spectrometry (M^+ = 1372).

Iodination of Bpa-hexarelin

Iodination of the photoactivatable derivative of hexarelin was performed in the dark by the lactoperoxidase method. Tyr-Bpa-Ala-hexarelin, 10 nmol, was mixed with 100 ng lactoperoxidase and 1 mCi $Na^{125}I$ in 40 µl sodium acetate buffer, 0.1 mol/l (pH 5.6). The reaction was started by adding 3 nmol H_2O_2. Incubation was carried out at 22°C for a total of 15 minutes, with the addition of a

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further 3 nmol H_2O_2 after 5 and 10 minutes. The radiolabelled peptide was purified by high-performance liquid chromatography on a Vydac C18 column using a 60-minute linear gradient of acetonitrile, 1 ml/minute, from 18 to 45% acetonitrile in 0.1% trifluoroacetic acid.

Membrane preparation

Human pituitaries were obtained from Ste-Justine Hospital, Montreal, Canada. Bovine and porcine pituitaries were provided by a local slaughterhouse. Human, bovine and porcine anterior pituitary membranes were prepared as described previously.[8] Briefly, pituitaries were homogenized at 4°C in Tris buffer containing protease inhibitors. The homogenate was centrifuged at 500 *g* for 10 minutes and the supernatant centrifuged at 20 000 *g* for 20 minutes. The resulting pellet was washed twice, resuspended in the homogenization buffer and frozen in liquid nitrogen.

Rat cardiac membranes were prepared as described by Harigaya and Schwartz.[9] Briefly, fresh rat hearts were homogenized at 4°C in 10 mmol $NaHCO_3$/l, containing 5 mmol NaN_3/l and 10 μmol Pefabloc/l. The homogenate was centrifuged at 8700 *g* for 20 minutes at 4°C. The supernatant was collected and the pellet resuspended in the same buffer; the resulting homogenate was centrifuged at 8700 *g* for 10 minutes at 4°C. The supernatant was pooled with the previous one and centrifuged at 35 000 *g* for 20 minutes at 4°C. The pellet was resuspended and homogenized in 20 mmol Tris–maleate/l (pH 6.8), containing 0.6 mol KCl/l. The homogenate was then centrifuged at 35 000 *g* for 60 minutes at 4°C. The resulting pellet was washed once in 10 mmol Tris–HCl/l (pH 7.4), resuspended in 50 mmol Tris–HCL/l (pH 7.4) containing 2 mmol EGTA/l, and frozen in liquid nitrogen.

Receptor binding and photolabelling with [^{125}I]Tyr-Bpa-Ala-hexarelin

Membrane preparations (400 μg) were incubated in the dark in 50 mmol Tris–HCl/l (pH 7.4), 2 mmol EGTA/l and 0.01% bacitracin in the presence of known concentrations of [^{125}I]Tyr-Bpa-Ala-hexarelin. For the covalent saturation binding studies, increasing concentrations (0.68, 1.3, 2.9, 7, 16 and 39 nmol/l) of the photoactivatable radioligand were used. Non-specific binding was determined by the addition of 10 μmol hexarelin/l.

For the competitive binding studies, increasing concentrations of the competitor (hexarelin or MK-0677; from 1 nmol/l to 10 μmol/l) were added simultaneously with the photoactivatable radioligand at 0.33 nmol/l. After an incubation period of 60 minutes at 22°C, membranes were irradiated with UV light (365 nm) for 15 minutes at 4°C. After centrifugation at 12 000 *g* for 10 minutes, pellets were resuspended in sample buffer (62 mmol Tris–HCl/l [pH 6.8], 2% sodium dodecylsulphate [SDS], 10% glycerol, 15% 2-mercaptoethanol and 0.05% bromophenol blue) and boiled for 5 minutes prior to SDS–polyacrylamide gel electrophoresis (SDS–PAGE; 7.5%). Autoradiographic exposure was carried out at –80°C using Dupont film with a reflection-intensifying screen.

RESULTS AND DISCUSSION

Biological activity of the photoactivatable hexarelin derivative

The biological activity of the photoactivatable hexarelin derivative was tested following its subcutaneous administration (300 μg/kg) to 10-day-old rats. GH levels were measured 15 minutes after injection. The hexarelin derivative elicited a 6.8-fold increase in plasma GH levels over the basal level (225 ± 29 vs 33 ± 3 ng/ml; $P < 0.001$), an increase comparable to that observed following administration of hexarelin at the same dose. Incorporation of the photoreactive group into hexarelin does not, therefore, alter its GH-releasing activity.

Covalent photolabelling of [^{125}I]Tyr-Bpa-Ala-hexarelin to GHRP receptors in pituitary membranes

The ability of the radiolabelled hexarelin derivative to bind irreversibly to its receptor was assessed in human, porcine and bovine anterior pituitary membrane preparations. Photolabelling of these membrane preparations revealed a major band on SDS–PAGE with an apparent molecular mass (M_r) of 57 000. The specificity of the photolabelled band was defined by the displacement of the signal in the presence of unlabelled hexarelin (10 μmol/l). When electrophoresis was carried out in non-reducing conditions, a major band at M_r 57 000 was still apparent, with no higher molecular weight band being visible, thus confirming that the GHRP receptor subtype consists of a single polypeptide chain of M_r 57 000. Deglycosylation produced only a slight shift in the M_r from 57 000 to 54 000, indicating that the GHRP receptor subtype has a low degree of glycosylation.

In order to evaluate the specificity and affinity of the binding of [^{125}I]Tyr-Bpa-Ala-hexarelin to the M_r 57 000 protein, covalent saturation studies were performed in which the GHRP receptor in bovine anterior pituitary membrane preparations was incubated with increasing amounts of the radioligand (from 0.68 to 39 nmol/l). Analysis of the resulting saturation curves showed a half-saturation of the ligand at 0.028 μmol/l, a concentration comparable to that reported in rat pituitaries obtained

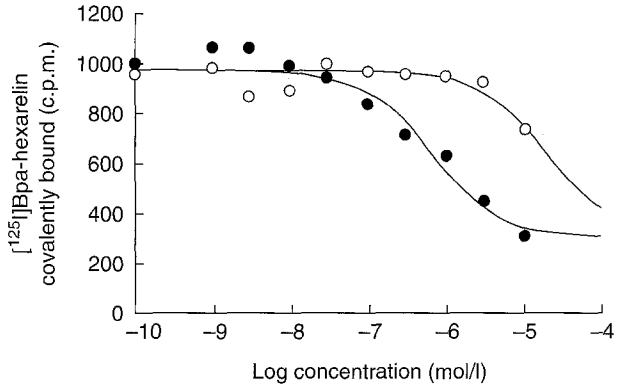

Fig. 1 Competitive binding studies of the photoactivatable hexarelin derivative with hexarelin or MK-0677 on bovine anterior pituitary membranes. [^{125}I]Tyr-Bpa-Ala-hexarelin (10^6 c.p.m.; 0.33 mmol/l) was incubated with bovine anterior pituitary membranes in the presence of increasing concentrations (1 nmol/l–10 μmol/l) of hexarelin (●) or MK-0677 (○) as the competitor. Following UV irradiation, 200 μg protein were subjected to SDS–PAGE. The incorporated radioligand was visualized using autoradiography, and the bands corresponding to M_r 57 000 were cut out and the radioactivity was measured using a gamma-counter. The resulting competition curves were evaluated by least-squares, non-linear regression analysis,[11] using the software ALLFIT for Windows.

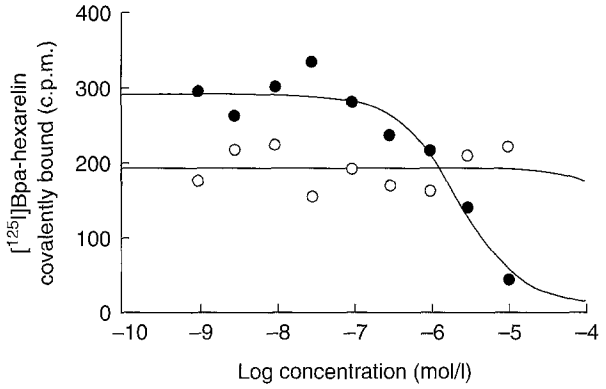

Fig. 2 Competitive binding studies of the photoactivatable hexarelin derivative with hexarelin or MK-0677 on rat cardiac membranes. [^{125}I]Tyr-Bpa-Ala-hexarelin (10^8 c.p.m.; 0.33 nmol/l) was incubated with rat cardiac membranes in the presence of increasing concentrations (1 nmol/l–10 μmol/l) of hexarelin (●) or MK-0677 (○) as the competitor. Following UV irradiation, 200 μg protein were subjected to SDS–PAGE. The incorporated radioligand was visualized using autoradiography, and the bands corresponding to M_r 57 000 were cut out and the radioactivity was measured using a gamma-counter. The resulting competition curves were evaluated by least-squares, non-linear regression analysis,[11] using the software ALLFIT for Windows.

from equilibrium binding studies.[10] Competitive binding studies with hexarelin or MK-0677 were also performed (Fig. 1). Hexarelin and MK-0677 were both found to compete with the photoactivatable radioligand for binding to the M_r 57 000 receptor, but to different degrees (50% inhibitory concentrations [IC_{50}] of 0.6 and 20 μmol/l, respectively).

The binding values reported here might be overestimated, due to the hydrophobicity of the GHRP derivatives. The hydrophobic nature of the peptides results in their partitioning in phospholipid membranes and thus affects their free concentrations during binding assays. This difference in affinity of hexarelin and MK-0677 for the M_r 57 000 receptor is in contrast to the finding that both compounds bind with equal affinity to the GH secretagogue receptor.[3] This suggests the existence of a GHRP receptor subtype that is distinct from the cloned receptor.

Covalent photolabelling of [^{125}I]Tyr-Bpa-Ala-hexarelin to GHRP receptors in cardiac membrane preparations

Analysis of the saturation curves obtained after incubating the GHRP receptor in rat cardiac membrane preparations with increasing concentrations of [^{125}I]Tyr-Bpa-Ala-hexarelin (from 1.5 to 45.0 nmol/l) gave a dissociation constant (K_d) of 14.5 nmol/l, and an estimate for the number of binding sites of 91 fmol/mg protein. Competition binding studies were performed to determine the specificity of the covalent photolabelling of the GHRP receptor in cardiac membrane preparations. Analysis of the competition binding curves of [^{125}I]Tyr-Bpa-Ala-hexarelin with hexarelin and MK-0677 as competitors gave IC_{50} values of 2.9 μmol/l and greater than 10 μmol/l, respectively, as shown in Fig. 2. The K_d obtained from the analysis of the saturation curve suggests that the radiolabelled photoactivatable ligand binds with higher affinity to the cardiac GHRP receptor than does hexarelin. However, the relative IC_{50} values for hexarelin and the spiroindoline derivative obtained from the competition binding curves allow us to conclude that the GHRP derivative binds specifically to the cardiac GHRP binding sites. The displacement profile of the radiolabelled photoactivatable derivative from cardiac membrane preparations by hexarelin and the spiroindoline derivative was different from that observed for pituitary membranes, suggesting that GHRP binding sites in the cardiovascular system are distinct from those found in the pituitary gland.

CONCLUSIONS

The existence of multiple GHRP receptor subtypes has been confirmed by a photoaffinity labelling approach using a photoactivatable derivative of hexarelin as the covalent ligand. At least two distinct GHRP receptor subtypes were detected specifically in pituitary and cardiac membranes, both of which are different from the recently cloned GH secretagogue receptor. The specific roles of both GHRP receptor subtypes in the regulation of

GH secretion and cardiovascular function have yet to be determined.

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