Specific receptors for synthetic GH secretagogues in the human brain and pituitary gland

G Muccioli, C Ghê, M C Ghigo, M Papotti¹, E Arvat², M F Boghen³, M H L Nilsson³, R Deghenghi⁴, H Ong⁵ and E Ghigo²

Department of Anatomy, Pharmacology and Forensic Medicine, ¹Department of Biomedical Sciences and Human Oncology, ²Department of Internal Medicine, University of Turin, Turin, Italy, ³Pharmacia and Upjohn, Stockholm, Sweden, ⁴Europeptides, Argenteuil, France, and ⁵Faculty of Pharmacy, University of Montreal, Montreal, Canada

(Requests for offprints should be addressed to G Muccioli, Department of Anatomy, Pharmacology and Forensic Medicine, Division of Pharmacology, University of Turin, Via P Giuria 13, 10125 Turin, Italy)

Abstract

In vitro studies have been performed to demonstrate and characterize specific binding sites for synthetic GH secretagogues (sGHS) on membranes from pituitary gland and different human brain regions. A binding assay for sGHS was established using a peptidyl sGHS (Tyr-Ala-hexarelin) which had been radioiodinated to high specific activity at the Tyr residue. Specific binding sites for ¹²⁵I-labelled Tyr-Ala-hexarelin were detected mainly in membranes isolated from pituitary gland and hypothalamus, but they were also present in other brain areas such as choroid plexus, cerebral cortex, hippocampus and medulla oblongata with no sex-related differences. In contrast, negligible binding was found in the thalamus, striatum, substantia nigra, cerebellum and corpus callosum. The binding of ¹²⁵I-labelled Tyr-Ala-hexarelin to membrane-binding sites is a saturable and reversible process, depending on incubation time and pH of the buffer. Scatchard analysis of the binding revealed a finite number of binding sites in the hypothalamus and pituitary gland with a dissociation constant (Kd) of (1.5 ± 0.3) × 10⁻⁹ and (2.1 ± 0.4) × 10⁻⁹ mol/l respectively. Receptor activity is sensitive to trypsin and phospholipase C digestion, suggesting that protein and phospholipids are essential for the binding of ¹²⁵I-labelled Tyr-Ala-hexarelin. The binding of ¹²⁵I-labelled Tyr-Ala-hexarelin to pituitary and hypothalamic membranes was displaced in a dose-dependent manner by different unlabelled synthetic peptidyl (Tyr-Ala-hexarelin, GHRP2, hexarelin, GHRP6) and non-peptidyl (MK 0677) sGHS. An inhibition of the specific binding was also observed when binding was performed in the presence of [d-Arg¹-d-Phe³-d-Trp⁷,⁹-Leu¹¹]-substance P, a substance P antagonist that has been found to inhibit GH release in response to sGHS. In contrast, no competition was observed in the presence of other neuropeptides (GHRH, somatostatin, galanin or Met-enkephalin) which have a known influence on GH release.

In conclusion, the present data demonstrate that sGHS have specific receptors in human brain and pituitary gland and reinforce the hypothesis that these compounds could be the synthetic counterpart of an endogenous GH secretagogue involved in the neuroendocrine control of GH secretion and possibly in other central activities.


Introduction

Growth hormone (GH) secretion is regulated by two neuropeptides: GH-releasing hormone (GHRH), which exerts stimulatory effects on GH secretion, and somatostatin, which exhibits an inhibitory influence (see Müller & Nisticò 1989 for review). GH release can also be stimulated by a group of synthetic oligopeptides termed growth hormone-releasing peptides (e.g. GHRP1, GHRP2, GHRP6 and its 2-methyl-d-Trp derivative hexarelin) which act through a mechanism different from that of GHRH (Bowers et al. 1991, Akman et al. 1993, see also Korbonits & Grossman 1995, Ghigo et al. 1997 for reviews). These synthetic GH secretagogues (sGHS), although modelled from Met-enkephalins, have lost the opioid activity and stimulate GH secretion of pituitary somatotroph cells both in vitro and in vivo in a variety of species, including rat, pig and human (Momany et al. 1981, Bowers et al. 1984, Bowers 1993, Deghenghi et al. 1994, Ghigo et al. 1994, Arvat et al. 1995). Since all these peptides have an oral bioavailability lower than 1%, a number of non-peptidyl sGHS (L-692,429, L-692,585 L-700,653 and MK 0677), with structures more amenable to chemical modification and optimization of oral bioavailability, have been developed and studied in both animals and man (Cheng et al. 1993, Gertz et al. 1993, Smith et al. 1993, Jacks et al. 1994, Chang et al. 1995). Among them, the spiroindoline derivative, MK 0677, proved to be the
strongest stimulator of GH secretion (Patchett et al. 1995). It allowed the identification and cloning of a specific G-protein-coupled receptor that mediates the activity of peptidyl and non-peptidyl sGHS (Howard et al. 1996). Such a receptor does not show significant homology with any other receptors known so far and it has been found expressed in the pituitary and hypothalamus of different mammalian species (Howard et al. 1996, Ponnès et al. 1996, Pong et al. 1996). These data supported the hypothesis that an endogenous sGHS-like ligand exists and is involved in the control of GH secretion (Bowers et al. 1991). In fact, the presence of GHS receptors (sGHS–R) in the pituitary gland and hypothalamus agrees with data for animals and humans showing that the GH-releasing activity of sGHS is the result of direct stimulation of somatotroph cells (Smith et al. 1993, Akman et al. 1993, Wu et al. 1994) and of a more important action at the hypothalamic level (Bowers et al. 1991, Fletcher et al. 1994, Fairhall et al. 1995, Hickey et al. 1996). The mechanisms of action underlying the GH-releasing activity of peptidyl and non-peptidyl sGHS seem to involve the antagonism of somatostatinergic pathways at both the pituitary and hypothalamic level as well as the stimulation of GHRH-secreting neurons (Conley et al. 1995, Hickey et al. 1996). Interestingly, the activity of sGHS is not fully specific since a slight stimulatory effect on prolactin, adrenocorticotrophin and cortisol levels as well as influences on the control of sleep and food intake have also been demonstrated (Jacks et al. 1994, Locke et al. 1995, Friebels et al. 1995, Arvat et al. 1997). These actions could take place via activation of specific receptors at levels other than the hypothalamo–pituitary system. In agreement with this hypothesis, peptidyl sGHS-binding sites have already been detected in the forebrain of rat and pig (Codd et al. 1988, Veeraragavan et al. 1992).

Based on the foregoing, the aim of the present research was to study the distribution and biochemical characterization of sGHS–R in the human pituitary gland and brain regions.

Materials and Methods

Chemicals

Hexarelin (His–d–2Me–Trp–Ala–Trp–d–Phe–Lys–NH₂) was kindly supplied by Pharmacia & Upjohn (Stockholm, Sweden). GHRP6 (His–d–Trp–Ala–Trp–d–Phe–Lys–NH₂), Tyr–Ala–hexarelin, GHRP2 (d–Ala–d–βNal–Ala–Trp–d–Phe–Lys–NH₂) and MK 0677 (N–[1(R)–[1,2-dihydro–1–methylasulphonylspiro–(3H–indole–3,4’–piperidin)–1’–yl]–2–(phenylmethoxy)–ethyl]–2–amino–2–methylpropanamide methanesulphonate) were supplied by Europeptides (Janke and Kunkel, Staufen, Germany) for 1 min at 4°C in 0·32 mol/l sucrose solution. After centrifugation at 500 g for 10 min the pellet was discarded and the supernatant centrifuged at 30 000 g for 30 min. The resulting pellet was resuspended in ice-cold buffer (50 mmol/l Tris–HCl, 100 G Muccioli and others · Receptors for synthetic GH secretagogues in humans


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Frozen pituitary gland and different brain regions were thawed and homogenized with an UltraTurrax T-25 (Janke and Kunkel, Staufen, Germany) for 1 min at 4°C and centrifuged at 30 000 g for 30 min. The resulting pellet was resuspended in ice-cold buffer (50 mmol/l Tris–HCl,
2 mmol/l EGTA, pH 7.3) and immediately used to determine protein content (Lowry et al. 1951) and for binding studies.

**Binding studies**

$^{125}$I-Labelled Tyr-Ala-hexarelin-binding studies were performed as previously described by Codd et al. (1989). For single point binding assay, tissue membranes (corresponding to 100 µg protein) were incubated in triplicate at 0°C for 60 min with approximately 0.5 $\times$ 10$^{-9}$ mol/l $^{125}$I-labelled Tyr-Ala-hexarelin in a final volume of 0.5 ml assay buffer (50 mmol/l Tris, 2 mmol/l EGTA, 0.1% BSA, 0.03% bacitracin, titrated with HCl to pH 7.3). Parallel incubations, where 2.5 $\times$ 10$^{-6}$ mol/l unlabelled Tyr-Ala-hexarelin was also present, were used to determine non-specific binding which was subtracted from total binding to yield specific binding values.

To establish binding site specificity, different sGHS (GHRP2, GHRP6, hexarelin, MK 0677) and various neuropeptides with a known effect on GH release such as GHRH, somatostatin, galanin, Met-enkephalin and [D-Arg$^9$-D-Phe$^5$-D-Trp$^{7,9}$-Leu$^{11}$]-substance P were also used in displacement assays with $^{125}$I-labelled Tyr-Ala-hexarelin. The binding reaction was terminated by adding 4 ml ice-cold assay buffer followed by rapid filtration over Whatman GF/B filters presoaked with 0.5% polyethyleneimine to prevent excessive binding of radioligand to the filters. Filters were rinsed 3 times with assay buffer and the radioactivity bound to membranes was measured by a Packard auto-gamma counter. A filter blank (binding of radioligand to glass fibre filters during the filtration procedure in the absence of membranes) was calculated for all incubations (between 3-5 and 4-5% of radioactivity added) and subtracted from the binding value. Only 1% of radioligand bound to blank filters was displaced by unlabelled Tyr-Ala-hexarelin. Thus tissues were considered to be sGHS-R-positive when a specific binding of 1% or more was observed. Specific binding was calculated as the difference between binding in the absence and presence of excess unlabelled Tyr-Ala-hexarelin and was expressed as a percentage of the total radioactivity added.

In some assays, receptor-binding saturation studies were conducted by incubating tissue membranes with increasing concentrations ((0.15–10) $\times$ 10$^{-9}$ mol/l) of radioligand in the absence and presence of a fixed amount (2.5 $\times$ 10$^{-6}$ mol/l) of unlabelled Tyr-Ala-hexarelin. Saturation isotherms were transformed using the method of Scatchard (1949) and the dissociation constant ($K_d$) and number of binding sites ($B_{max}$) were calculated with the GraphPAD Inplot program (GraphPAD Software, San Diego, CA, USA).

**Statistical analysis**

Data are expressed as means ± S.E.M. unless otherwise noted. Significant differences between groups were assessed by one-way ANOVA followed, when appropriate, by Duncan’s multiple range test (Steel & Torrie 1960).

**Results**

**Binding of $^{125}$I-labelled Tyr-Ala-hexarelin to membranes from human brain and pituitary gland**

The results of a survey of specific binding of $^{125}$I-labelled Tyr-Ala-hexarelin to membranes from pituitary gland and different brain regions of adult male and female subjects.
Binding varied greatly with the tissue examined. The highest $^{125}\text{I}$-labelled Tyr-Ala-hexarelin binding activity was observed in the hypothalamus and pituitary gland of both sexes. Tyr-Ala-hexarelin binding was recorded in all specimens of these tissues, with binding values ranging from 11·2 to 17·4% for hypothalamus (11·2–15·0% for males and 11·2–17·4% for females) and from 11·0 to 14·9% for pituitary gland (11·0–14·9% for males and 11·3–14·0% for females). The specific binding in the hypothalamus or pituitary gland represented 52–67% of total radioactivity bound. In addition, specific binding was also clearly present in the choroid plexuses, cerebral cortex, hippocampus and medulla oblongata, with values that were about one half of those found in the hypothalamus and pituitary gland. Other brain regions tested, such as thalamus, striatum, substantia nigra, cerebellum and corpus callosum, did not exhibit significant binding of $^{125}\text{I}$-labelled Tyr-Ala-hexarelin. Differences in the specific binding values were statistically significant for the hypothalamus, pituitary gland, choroid plexuses, cerebral cortex, hippocampus and medulla oblongata vs the other brain regions ($P<0·001$ in both sexes) and for hypothalamus and pituitary gland vs choroid plexuses, cerebral cortex, hippocampus and medulla oblongata ($P<0·01$ in both sexes). Specific binding of $^{125}\text{I}$-labelled Tyr-Ala-hexarelin to membranes from pituitary gland or different brain regions was not correlated with the cause of death and the time elapsed from death to autopsy (23–48 h) in both sexes. Moreover, at histopathologic examination, tissues were all well preserved both from an architectural and cytological point of view.

**Biochemical characteristics of binding**

To determine whether the binding of $^{125}\text{I}$-labelled Tyr-Ala-hexarelin to tissue membranes shows the properties typical of ligand–receptor interaction, the binding of radiotracer was investigated in more detail in tissues (hypothalamus or pituitary gland) that displayed the highest levels of specific binding. As shown in Fig. 1a, the specific binding of $^{125}\text{I}$-labelled Tyr-Ala-hexarelin to membranes of these tissues increased with the time of incubation, reaching equilibrium after 60 min at 0°C and declining slowly after 90 min of incubation. The specific binding of radiolabelled Tyr-Ala-hexarelin to pituitary and hypothalamic membranes was reversible; once formed, the complex tracer–membranes could be quickly dissociated by adding a large excess ($2·5 \times 10^{-5}$ mol/l) of unlabelled Tyr-Ala-hexarelin. About 50% of radiotracer specifically bound to the pituitary and hypothalamic membranes was dissociated within 60 min at 0°C (Fig. 1b).

A study of specific binding as a function of membrane protein concentration indicated that the binding was proportional to the protein up to at least 100 µg/tube. Therefore all our incubations were performed with about 100 µg protein/tube (Fig. 2). Specific binding of $^{125}\text{I}$-labelled Tyr-Ala-hexarelin to pituitary gland and hypothalamic membranes occurred over a relatively wide range of pH. The maximal binding was observed at pH 5·6 and declined to half-maximal values at pH 4·5 and 8·6. However, varying the pH of the incubation from pH 5·6...
to 7.3 did not alter the binding significantly. Therefore the experiments were carried out at pH 7.3 (Fig. 3).

Brief exposure (5 min) of pituitary membranes to enzymes that disrupt protein structure such as trypsin (50 μg/ml) resulted in a marked decrease (−68%) in the specific binding of radiolabelled Tyr-Ala-hexarelin, suggesting that protein is a functionally important part of the binding site. Phospholipase C (50 μg/ml) also caused a significant decrease (−28%) in binding, suggesting that phospholipids may also play an important role in the binding of Tyr-Ala-hexarelin.

Experiments using increasing concentrations of radioiodinated Tyr-Ala-hexarelin ranging from 0.15 × 10⁻⁹ to 10 × 10⁻⁹ mol/l revealed evidence of saturable specific binding in the hypothalamus of three male subjects which yielded sufficient amounts of tissue membranes (Fig. 4).
Scatchard analysis (upper panel Fig. 4) indicated the presence of a single class of high-affinity sites with a \( K_d \) of \( (1.5 \pm 0.3) \times 10^{-9} \) mol/l and a \( B_{\text{max}} \) of 1110 \( \pm 123 \) fmol/mg protein. Similar specific high-affinity binding was also detected in pituitary membranes of the same subjects, indicating a \( K_d \) of \( (2.1 \pm 0.4) \times 10^{-9} \) mol/l and a \( B_{\text{max}} \) of 963 \( \pm 172 \) fmol/mg protein.

**Specificity of binding**

The specificity of \( ^{125}\text{I} \)-labelled Tyr-Ala-hexarelin binding was established by determining the ability of different compounds to compete with the radioligand for the hypothalamic binding sites (Fig. 5). Both unlabelled peptidyl (Tyr-Ala-hexarelin, hexarelin, GHRP2, GHRP6) and non-peptidyl (MK 0677) sGHS completely displaced radiolabelled Tyr-Ala-hexarelin from binding sites, but MK 0677 was significantly less potent than peptidyl sGHS. The IC\(_{50}\) values (mean \( \pm \) S.E.M. of three separate experiments) were \((1.2 \pm 0.1) \times 10^{-8} \) mol/l for Tyr-Ala-hexarelin, \((1.6 \pm 0.2) \times 10^{-8} \) mol/l for GHRP2, \((1.8 \pm 0.2) \times 10^{-8} \) mol/l for hexarelin, \((2.4 \pm 0.3) \times 10^{-8} \) mol/l for GHRP6 and only \((1.1 \pm 0.3) \times 10^{-7} \) mol/l for MK 0677. [\( \text{d-Arg}^1\text{-d-Phe}^5\text{-d-Trp}^7\text{-Leu}^{11} \)]-peptide, a substance P antagonist that has been found to inhibit the GH release in response to sGHS both in vivo and in vitro (Bitar et al. 1991, Cheng et al. 1994), also displaced this binding, but its IC\(_{50}\) value was about 3 times higher than that of MK 0677. In contrast, no competition was observed in the presence of other neuropeptides (GHRH, somatostatin, galanin or Met-enkephalin) that have known effects on GH release. The pattern of displacement specificities in the pituitary gland and other sGHS-R-positive brain areas resembled that of the hypothalamus (data not shown).

**Discussion**

In the present study, we have demonstrated the properties of specific receptors for sGHS in the human brain and pituitary gland. sGHS-Rs have been identified by radioreceptor analysis using a peptidyl sGHS (Tyr-Ala-hexarelin) which had been radiiodinated at high specific activity (\( > 2000 \) Ci/mmol) at the Tyr residue. The highest specific binding of \( ^{125}\text{I} \)-labelled Tyr-Ala-hexarelin was found in the hypothalamus and pituitary gland and a lower but significant binding was also detected in the choroid plexuses, cerebral cortex, hippocampus and medulla oblongata, whereas negligible binding was observed in the striatum, substantia nigra, thalamus, cerebellum and corpus callosum. No sex differences in radiolabelled Tyr-Ala-hexarelin binding to either pituitary gland or brain regions were observed, suggesting that sGHS-Rs were not influenced by sex hormones. This finding is in agreement with previous evidence showing that the GH-releasing activity of sGHS is not sex dependent (Bowers 1993, Ghigo et al. 1994).

\( ^{125}\text{I} \)-Labelled Tyr-Ala-hexarelin binding shows many of the properties typical of ligand–receptor interaction (Reichert 1988). These include high affinity, saturability and reversibility. As described for other peptides (Furchgott 1978, Reichert 1988), the rate and extent of binding increased if the concentration of either radioligand or receptors was increased. \( ^{125}\text{I} \)-Labelled Tyr-Ala-hexarelin binding was also dependent on time of incubation, and exhibited an optimum pH. Moreover, loss of receptor activity caused by trypsin and phospholipase C treatments indicates that protein and phospholipid constituents are structural components that are essential for receptor activity. The binding specificity of \( ^{125}\text{I} \)-labelled Tyr-Ala-hexarelin to pituitary or hypothalamic membranes is very similar to that observed in the rat and pig tissues (Sethumadavan et al. 1991, Veeraragavan et al. 1992, Pomés et al. 1996, Pong et al. 1996): radioligand binding is displaced by unlabelled peptidyl sGHS (Tyr-Ala-hexarelin, hexarelin, GHRP2, GHRP6), non-peptidyl sGHS (MK 0677) and by a substance P antagonist such as [\( \text{d-Arg}^1\text{-d-Phe}^5\text{-d-Trp}^7\text{-Leu}^{11} \)]-peptide, but not by other neuropeptides (GHRH, galanin, somatostatin or Met-enkephalin) that have a known effect on GH release. The displacement of \( ^{125}\text{I} \)-labelled Tyr-Ala-hexarelin by [\( \text{d-Arg}^1\text{-d-Phe}^5\text{-d-Trp}^7\text{-Leu}^{11} \)]-peptide parallels its antagonist actions on GH secretion, since this peptide has been found to selectively inhibit GH release in
response to non-peptidyl and peptidyl sGHS both in vivo and in vitro (Bitar et al. 1991, Cheng et al. 1994). Our competition experiments also demonstrate that the different peptidyl sGHS studied here showed a similar efficacy in displacing 125I-labelled Tyr-Ala-hexarelin, whereas MK 0677 was about 5–9 times less effective than these molecules. This agrees with previous clinical studies showing that hexarelin, GHRP2 and GHRP6 stimulate GH secretion in vivo with equal potency and that they are clearly more active than MK 0677 (Korbonits & Grossman 1995, Arvat et al. 1997, Ghigo et al. 1997).

The presence of specific sGHS-R in the human pituitary strengthens the hypothesis that this tissue may be an important target of sGHS. This is in agreement with other data in humans showing that these synthetic compounds release GH from the pituitary gland (Renner et al. 1994, Lei et al. 1995). Our observation that there are considerable amounts of sGHS-R in the hypothalamus indicate that this region may be a further target of sGHS. This agrees with other data showing that sGHS activate in GH-deficient rats and mice distinct subpopulations of hypothalamic arcuate neurones, as reflected by increased electrical activity (Dickson et al. 1995) and by the detection of Fos protein in cell nuclei (Dickson et al. 1995, Sirinathsinghi et al. 1995). More recently, evidence has been presented that the arcuate neurones activated by systemic or intracerebroventricular injections of GHRP6 are mainly of the neurosecretory type and contain GHRH or neuropeptide Y (Dickson et al. 1996, Dickson & Luckman 1997).

The present data also show, for the first time, the presence of sGHS-R in the choroid plexuses. As reported for other peptides (Pardridge 1986), these binding sites may act as transport proteins, translocating peptidyl sGHS from the blood to the cerebrospinal fluid. This mechanism could serve to transport sGHS molecules through the blood–brain barrier and should allow their interaction with other brain areas such as cerebral cortex, hippocampus and medulla oblongata, where we found considerable amounts of sGHS-R.

In conclusion, the present data demonstrate that sGHS have specific receptors in human pituitary and brain and reinforce the hypothesis that these compounds could be the synthetic counterpart of an endogenous GH secretagogue involved in the neuroendocrine control of GH secretion and possibly in other central activities.

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