Growth hormone-releasing hexapeptide (GHRP-6) increases intracellular calcium concentrations in cultured cells from human pituitary adenomas of different types

Andrea Lania, Emilia Ballaré, Sabrina Corbetta, Marcello Filopanti, Luca Persani and Anna Spada

Institute of Endocrine Sciences, Maggiore Hospital IRCCS, University of Milan, Italy and Italian Auxological Institute IRCCS, Milan, Italy

(Correspondence should be addressed to A Spada, Institute of Endocrine Sciences, University of Milan, Ospedale Maggiore IRCCS, via F. Sforza 35, 20122 Milan, Italy)

Abstract

Objective: The GH-releasing peptide GHRP-6, has been found to interact with specific receptors in somatotrophs, causing cytosolic Ca$^{2+}$ ([Ca$^{2+}$]$_i$) rise and GH release. Moreover, this peptide has been demonstrated to stimulate the secretion of pituitary hormones other than GH, i.e. ACTH and prolactin, this effect being generally attributed to a central action. In this study we evaluated whether the pituitary action of this peptide is restricted to cell type of somatotroph lineage.

Methods: The effect of GHRP-6 on [Ca$^{2+}$]$_i$ was tested in cell preparations obtained from a series of human pituitary adenomas (9 GH-secreting adenomas, 7 nonfunctioning adenomas, 3 ACTH-secreting adenomas, 2 TSH-secreting adenomas and 1 prolactinoma) loaded with the Ca$^{2+}$ indicator fura-2.

Results: GHRP-6, at concentrations higher than 1 nmol/l, significantly increased [Ca$^{2+}$]$_i$ in all tumours, with the exception of the 3 ACTH-secreting adenomas in which the peptide was ineffective at any concentration tested (from 1 nmol/l to 1 µmol/l). By contrast, in all ACTH-secreting adenomas, both corticotrophin-releasing hormone and pituitary adenylate cyclase activating peptide caused a marked [Ca$^{2+}$]$_i$ increase. In tumours responsive to GHRP-6, the peptide caused a typical biphasic [Ca$^{2+}$]$_i$ rise due to Ca$^{2+}$ mobilization from the intracellular stores and Ca$^{2+}$ influx through voltage-dependent Ca$^{2+}$ channels.

Conclusions: These data indicate that almost all tumoral pituitary cell types are targets of GHRP-6 action, the only exception being corticotrophs.

European Journal of Endocrinology 139: 343–348

Introduction

The secretion of growth hormone (GH) is under the complex control of both stimulatory and inhibitory agents. Although evidence indicates that growth hormone-releasing hormone (GHRH) is the physiological stimulator of GH secretion, other peptides have been demonstrated to influence GH secretion both in vivo and in vitro. In particular, synthetic peptides derived from enkephalins, such as the GH-releasing peptide, GHRP-6, have been found specifically to stimulate GH secretion both in animals and in humans (1–6). Although endogenous GHRPs have not yet been identified, the presence of peptides with GH releasing properties has been further suggested by the recent cloning of a G-protein-coupled receptor specifically activated by GHRPs and expressed in the pituitary and hypothalamus (7–9). The action of GHRP-6 seems to be mediated via activation of phospholipase C and phosphoinositide breakdown, together with increased Ca$^{2+}$ influx through voltage-dependent calcium channels (1, 10–12). In addition to the stimulatory action at the hypothalamic level, in vitro studies with synthetic GHRP-6 have demonstrated that this compound may act specifically on somatotrophs to stimulate GH release (10, 12). Moreover, it has been observed that GHRPs may stimulate the secretion of other pituitary hormones – adrenocorticotropic hormone (ACTH) and prolactin (PRL) – this effect being generally attributed to a central action of these compounds (1, 13–16).

It is well established that human pituitary adenomas express receptors for a large number of hypothalamic neuropeptides (17). In particular, GHRH is effective in increasing the production of cyclic AMP and the in vitro release of GH from 50% of GH-secreting adenomas (18), the lack of GHRH effect being, at least in part, due to mutations of the Gs$^{a}$ gene, leading to constitutive activation of adenylyl cyclase (19). Similarly, these tumours are sensitive to GHRP-6, as this agent has been reported to induce an increase in the formation of inositol 1,4,5-trisphosphate (IP$_3$) and intracellular calcium concentrations in tumoral somatotrophs (20).
Data on the sensitivity of pituitary tumours other than GH-secreting adenomas to GH-releasing peptides are not available in the literature. In order to evaluate whether GHRP-6 action is specific for the cell type of the somatotroph lineage, a series of human pituitary adenomas characterized by different cell composition was tested for sensitivity to GHRP-6. Results indicate that GHRP-6 increases Ca\(^{2+}\) mobilization and influx in almost all secreting and non-secreting tumours, the only exception being corticotroph adenomas.

**Materials and methods**

**Materials**

GHRP-6 and pituitary adenylate cyclase activating peptide-38 (PACAP) were obtained from Peninsula Labs (St Helens, Merseyside, UK). Corticotrophin-releasing hormone (CRH), GHRH, thyrotrophin-releasing hormone (TRH), gonadotrophin-releasing hormone (Grh), somatostatin, trypsin and soybean trypsin inhibitor were purchased from Sigma (St Louis, MO, USA). Nimodipine and nifedipine were obtained from Tocris Cookson, Bristol, UK. Culture media were purchased from Flow Laboratories (Mackenheim, Germany). Hexarelin, a GHRP-6 analogue, was a kind gift from Mediolanum Pharmaceuticals (Milan, Italy). Fura-2-AM was purchased from Molecular Probes (Junction City, OR, USA). All other reagents were reagent grade.

**Adenomas**

We studied nine GH-secreting adenomas (GH-omas; negative for mutations in the Gs\(_\alpha\) gene), seven non-functioning adenomas (NFP A), three ACTH-secreting adenomas (ACTH-omas), two thyroid-stimulating hormone (TSH)-secreting adenomas (TSH-omas) and one macroadenoma. The presence of tumours of different types was diagnosed on the basis of clinical features and standard hormonal criteria and confirmed after surgery by morphological analyses. No patient had previously undergone pituitary irradiation. Small adenoma fragments were fixed for immunohistochemistry to check the nature of the material, as previously described (21), and the remaining tissue was placed in sterile culture medium for cell culture.

**Cell culture**

Cells were enzymatically dispersed using trypsin and DNase as previously described (22). The suspension obtained consisted largely of single cells with a viability, as assessed by trypan blue exclusion, greater than 90%. Cells were maintained in suspension in bacteriological dishes at a density of 5 \(\times\) 10\(^5\) cell/ml in Dulbecco’s Modified Essential Medium (DMEM) supplemented with 10% fetal calf serum and antibiotics at 37 °C and in an atmosphere of 5% CO\(_2\). After 24 h, the medium was removed and stored at −20°C until required for GH assay, and cells were collected in Krebs–Ringer–Hepes (KRH) incubation medium for measurement of cytosolic Ca\(^{2+}\). GH was measured in cell supernatants by an immunofluorimetric assay using a commercial kit (Pharmacia, Turku, Finland).

**Measurement of cytosolic free Ca\(^{2+}\)**

After 24 h in DMEM, cells were used for measurement of [Ca\(^{2+}\)]\(_i\), as previously described (23). Briefly, cells were resuspended at 4–5 \(\times\) 10\(^6\) cells/ml in KRH incubation medium containing: 125 mmol/l NaCl, 5 mmol/l KCl, 1.2 mmol/l KH\(_2\)PO\(_4\), 1.2 mmol/l MgSO\(_4\), 2 mmol/l CaCl\(_2\), 25 mmol/l Hepes–NaOH (pH 7.4) and 6 mmol/l glucose. Cells were loaded with the Ca\(^{2+}\) indicator, fura-2, by incubation with 5 \(\mu\)mol/l fura-2-acetoxymethylester for 30 min at 37°C. Fluorescence recordings were carried out with a cell concentration of 3–4 \(\times\) 10\(^6\)/ml in a Perkin Elmer LS5 spectrofluorimeter (Perkin Elmer, Norwalk, CT, USA) at 345 nm excitation and 490 nm emission, with slits of 5 and 10 nm, respectively. [Ca\(^{2+}\)]\(_i\) was calculated according to the method of Gryeukievicz et al. (24) All values were corrected for changes in autofluorescence (23).

**Statistical analyses**

The results are expressed as means ± s.d. Paired two-tailed Student’s t-test or one-way ANOVA were used to detect the significance between two series of data where appropriate. \(P < 0.05\) was accepted as statistically significant.

**Results**

**Adenomas**

The morphological characteristics of the adenomas included in the present series were evaluated by immunocytochemistry and were consistent with the clinical and biochemical parameters of the individual patients. By immunofluorescence, all GH-, ACTH- and TSH-secreting adenomas showed positivity for GH, ACTH and TSH respectively, 50–90% of the total cells being positive, whereas all NFP A were positive for at least one glycoprotein. In tumours other than GH-omas immunoreactivity for GH was either absent or present only in a small number of cells (<5% of total cells, data not shown). Morphological data of NFP A have been reported previously elsewhere (25).

**Effect of GHRP-6 on cytosolic Ca\(^{2+}\) concentrations**

GHRP-6 was effective in modifying cytosolic [Ca\(^{2+}\)]\(_i\), in the majority of pituitary tumours. In particular,
tumoral cells obtained from all GH-omas (n = 9), NFPA (n = 7), TSH-omas (n = 2) and one prolactinoma showed a significant increase in [Ca^{2+}], after the addition of 100 nmol/l GHRP-6 (Fig. 1). In contrast, none of the ACTH-omas tested (n = 3) was sensitive to this agent. The increases induced by GHRP-6 varied from one adenoma to another, but not within the same cell preparation. The effectiveness of GHRP-6 in increasing [Ca^{2+}], was similar in the different types of adenomas, with the exception of ACTH-omas (Fig. 1). In the responsive tumours, GHRP-6 was effective at concentrations greater than 1 nmol/l and the maximal increase occurred at 100 nmol/l.

GHRP-6 applied to a Ca^{2+}-containing medium caused a typical biphasic increase in [Ca^{2+}], comprising a rapid increase, followed by a plateau that was sustained for many minutes (Fig. 2). A similar increase in [Ca^{2+}], was observed after the addition of the GHRP-6 analogue, hexarelin (10 nmol/l), in the five tumours tested (three GH-omas and two NFPA; data not shown). The initial peak triggered by GHRP-6 was maintained in calcium-free medium, indicating that this component of the changes in [Ca^{2+}], was due to mobilization of Ca^{2+} from the intracellular stores (Fig. 2). The blockade of voltage dependent Ca^{2+} channels by dihydropyridine antagonists, such as nimodipine and nifedipine, caused a rapid decrease in resting [Ca^{2+}], values (Fig. 2). Moreover, these agents reduced the transient changes attributable to Ca^{2+} mobilization and completely abolished the plateau phase caused by the stimulation of Ca^{2+} influx through voltage-dependent Ca^{2+} channels induced by GHRP-6 (Fig. 2). A similar effect was observed in two GH-omas and one NFPA when Ca^{2+} influx was blocked by the addition of somatostatin (100 nmol/l) (data not shown).

Experiments were carried out to rule out the possibility that the responsiveness to GHRP-6 observed in tumours other than GH-omas might be due to the presence of somatotrophs in the tumour cell preparations. Whereas, in all GH-omas, GHRH increased [Ca^{2+}], by stimulating Ca^{2+} influx from the extracellular milieu, this effect was not observed in any other tumours of this series (Fig. 3). Moreover, the release of GH measured in media collected from tumours other than GH-omas was very low or undetectable (<0.01 compared with 710 ± 280 μg/10^6 cells).

GHRP-6 was not the only peptide able to modulate [Ca^{2+}], in tumours of different types. Among the six NFPA tested for the sensitivity to different peptides, TRH (100 nmol/l) increased [Ca^{2+}], in five tumours, PACAP-38 (100 nmol/l) increased it in four and GnRH (100 nmol/l) increased it in three (Figs 3, 4). Similarly, both TRH and PACAP increased [Ca^{2+}], in cells obtained from the two TSH-omas (data not shown). Although, in all ACTH-omas, GHRP-6 was ineffective in increasing
[Ca$^{2+}$], at any concentration tested in the range 1 nmol/l to 1 μmol/l (Figs 1, 5), in these tumours both CRH and PACAP caused a marked increase in [Ca$^{2+}$]. The effect of CRH (increase over basal value from 70% to 110%) was mainly due to intracellular mobilization of Ca$^{2+}$, as the effect was maintained in Ca$^{2+}$-free medium, while the effect of PACAP was largely dependent on extracellular Ca$^{2+}$ (Fig. 5, and data not shown).

**Discussion**

This study is the first to indicate that GHRP-6 directly modulates intracellular effectors in several pituitary cell types. In particular, GHRP-6 elicited a clear increase in [Ca$^{2+}$], in somatotrophs and lactotrophs, and in cells synthesizing glycoproteins. The number of tumours included in the study was insufficient to permit us to draw conclusive information for some cell types: in particular, because medical treatment is the first choice treatment of patients with prolactinomas in our institution, only one prolactinoma was available for in vitro studies. Similarly, because of the rarity of the disease, only two TSH-secreting adenomas were studied. However, taken as a whole, the results obtained in pituitary tumours of different types seem to be in contrast with the putative concept of selectivity of GHRP-6 action in the pituitary.

The effect of GHRP-6 on [Ca$^{2+}$], recorded in cells from NFPA, TSH-omas and PRL-oma was similar, in terms of efficacy and potency, to that observed in GH-omas and consistent with results reported in the normal rat pituitary (11, 12). In these tumours, increases in [Ca$^{2+}$], induced by both GHRP-6 and the analogue, hexarelin, were found to have a dual origin: mobilization from intracellular stores and influx from the extracellular spaces. Taking into account that the increase in intracellular mobilization of Ca$^{2+}$ is due to the formation of IP$_3$ during receptor activation, the pattern of increase in [Ca$^{2+}$], induced by GHRP-6 in cells obtained from pituitary tumours is consistent with the mechanism of action of GHRP-6, which involves activation of phospholipase C and inositol phospholipid turnover (1, 10–12). Moreover, in agreement with observations in normal rat pituitary (11, 12), GHRP-6 increased the influx of Ca$^{2+}$ through voltage dependent calcium channels, as indicated by the effects of the
dihydropyridine antagonists, nimodipine and nifedipine. Although it is established that the increase in Ca\(^{2+}\) caused by GHRP-6 in GH-omas stimulates in vitro release of GH (20, and personal observation), from our study it is not possible to infer the effect of GHRP-6 on hormonal secretion from tumours other than GH-omas.

The possibility that the effects of GHRP-6 observed in pituitary tumours might be due to the presence of somatotrophs contaminating cell preparations seems to be unlikely, as GHRH was ineffective in modifying [Ca\(^{2+}\)]\(_i\) in cells obtained from tumours other than GH-omas. This conclusion is consistent with the immunofluorescence and in vitro secretion data indicating a very low, if any, presence of somatotrophs in tumours derived from other cell types. Moreover, these data confirm and extend the observation that the expression of GHRH receptors is restricted to normal and tumoral somatotrophs (17).

From our experiments, it is not possible to elucidate whether the sensitivity of several cell types to GHRP-6 depends on tumoral transformation. In fact, receptors for hypothalamic agents that are ineffective in normal cell counterparts are frequently expressed by pituitary tumours, and by this mechanism tumours acquire an abnormal susceptibility to stimulatory or inhibitory inputs, or both (17, 26, 27). Very recently, GHRP-6 receptor mRNA has been found in different types of pituitary adenomas, although the actual percentage of tumours, particularly of NFPA, expressing this receptor remains controversial (28, 29). However, as cell types expressing GHRP-6 receptors in the normal pituitary have not yet been clearly identified, it is difficult to ascertain whether the increase in Ca\(^{2+}\) induced by GHRP-6 in pituitary tumours results from the normal or abnormal expression of GHRP-6 receptors.

At variance with the sensitivity to GHRP-6 displayed by tumoral somatotrophs, lactotrophs, gonadotrophs and thyrotrophs, GHRP-6 was totally ineffective in cells obtained from the three ACTH-omas studied. Although the lack of effect of GHRP-6 on [Ca\(^{2+}\)]\(_i\), might have been due to either receptor or postreceptor defects, the most likely explanation for this finding is a lack of expression of GHRP-6 receptors in this cell type. In fact, in the same cell preparations, the specific releasing hormone, CRH, was able to increase [Ca\(^{2+}\)]\(_i\), efficiently by stimulating Ca\(^{2+}\) mobilization and influx, as already reported (30). Similarly, in agreement with the demonstration that ACTH-omas generally express PACAP receptors (31), this peptide induced increases in [Ca\(^{2+}\)]\(_i\) in all tumours tested. These observations are consistent with the general view that the increase in circulating ACTH concentrations induced by GHRP-6 analogues in healthy subjects might be due to an indirect action of these compounds at the hypothalamic level (13).

Taken together, these data indicate that, in addition to TRH and PACAP, which are known to be general activators of second messengers in pituitary tumours, GHRP-6 also activates intracellular effectors in all tumoral pituitary cell types, with the single exception of corticotrophs.

**Acknowledgements**

This work was partially supported by the grant 9706151106 from MURST (Rome) and grants from Ospedale Maggiore IRCCS (Milan), and the Auxological Italian Institute IRCCS (Milan). We would like to thank Drs G Faglia and P Beck-Peccoz for critical reading of the manuscript. We are indebted to Drs M Giovanelli, P Mortini and M Losa (Department of Neurosurgery, Scientific Institute San Raffaele, Milan), and G P Tonnarelli (Department of Neurosurgery, Legnano Hospital, Legnano) for the supply of pituitary adenomas.

**References**


intracellular signaling, with a focus on the role of growth hormone releasing peptide (GHRP-6) and its synergistic effects with growth hormone releasing hormone (GHRH). The study aimed to clarify the mechanisms by which GHRP-6 enhances GH secretion in patients with GH deficiency, particularly those with hypothalamic-pituitary dysfunctions.

The study's findings supported the hypothesis that GHRP-6 acts as an agonist at the GHRP-1 receptor, which is distinct from the GHRH receptor, and has a unique role in GH release. The authors concluded that GHRP-6 may represent a new therapeutic approach for the treatment of GH deficiency, particularly in cases where traditional GH replacement therapy is insufficient. The study's results have implications for future research and clinical applications in the management of GH deficiency and related conditions.