Cortistatin mimics somatostatin by inducing a dual, dose-dependent stimulatory and inhibitory effect on growth hormone secretion in somatotropes

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Abstract

Cortistatin is a recently discovered neuropeptide that is structurally related to somatostatin, the classic inhibitor of growth hormone (GH) release. Cortistatin binds with high affinity to all five somatostatin receptors (sst1–5), and, like somatostatin, cortistatin inhibits *in vivo* GH release in man and rats. In this report, we compared the *in vitro* actions of cortistatin and somatostatin using primary pig pituitary cell cultures. In this species, we have previously reported that somatostatin not only inhibits GH-releasing hormone (GHRH)-stimulated GH release at high doses, but also stimulates basal GH release at low (pM) doses, a dual response that is markedly dependent on the subpopulation of pituitary somatotropes examined. Results reported herein demonstrate that cortistatin closely mimics the dose-dependent inhibitory and stimulatory effects of somatostatin on GH secretion. As cortistatin, unlike somatostatin, binds to the human receptor for ghrelin/GH secretagogues (GHS-R), we also investigated whether cortistatin stimulates GH release through this receptor by using a synthetic, short form of cortistatin, cortistatin-8 (CST8), which lacks the sst-binding capacity of full-length cortistatin but retains its GHS-R-binding capacity. Interestingly, CST8 stimulated GH release only at low doses (10^{-15} M), and did not reduce GH secretion stimulated by GHRH, ghrelin, or low-dose, full-length cortistatin, yet it counteracted that induced by a nonpeptidyl GHS, L-163 255. Taken together, our results indicate that the dual, inhibitory and stimulatory effects of cortistatin on GH release closely parallel those of somatostatin and are probably mediated by the same receptor(s) and signaling pathway(s) for both peptides. Furthermore, they suggest that the pathway(s) activated by cortistatin (and somatostatin) to stimulate GH release are not initiated by GHS-R activation.

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Introduction

Cortistatin is a peptide originally cloned from neuronal tissues of man, rats and mice (de Lecea *et al.* 1996, 1997a, Fukusumi *et al.* 1997, Spier & de Lecea 2000). Cortistatin is expressed as a pre-pro-peptide in various brain regions, including cortex, hippocampus and hypothalamus (de Lecea *et al.* 1996, 1997b, Spier & de Lecea 2000) and in multiple peripheral tissues (de Lecea *et al.* 1997a, Spier & de Lecea 2000, Dalm *et al.* 2004), and it has been recently shown to be present in various human neuroendocrine tissues and related tumors, including the pituitary (Allia *et al.* 2005). Pre-pro-cortistatin is structurally homologous to pre-pro-somatostatin, with high similarity in the carboxyl terminus from which both peptides are processed (Fukusumi *et al.* 1997, Spier & de Lecea 2000); however, each peptide is encoded by a distinct gene (de Lecea *et al.*

1996, Fukusumi *et al.* 1997, Spier & de Lecea 2000). Human pre-pro-cortistatin may be cleaved to pro-CST, from which two mature products, CST-17 and CST-29, can be generated in man (Fukusumi *et al.* 1997, de Lecea *et al.* 1997a, Spier & de Lecea 2000). CST-17 shares 10 of the 14 amino-acid residues with somatostatin 14.

Owing to their high structural similarity, cortistatin binds all somatostatin receptors (sst1–5) with an affinity close to that of somatostatin and therefore was expected to have similar biologic activities (Fukusumi *et al.* 1997, Siehler *et al.* 1998, Spier & de Lecea 2000). However, recent studies have shown that not all of the actions of cortistatin are shared with those of somatostatin, suggesting the existence of receptors for cortistatin distinct from sst (de Lecea *et al.* 1996, Siehler *et al.* 1998, Sánchez-Alavez *et al.* 2000, Spier & de Lecea 2000, de Lecea 2005, Spier *et al.* 2005). This hypothesis has been also supported by the finding that an orphan

G-protein-coupled receptor, MrgX2, can selectively bind cortistatin, but not somatostatin (Robas *et al.* 2003). However, MrgX2 was found in a recent study to bind with higher affinity to proadrenomedullin N-terminal peptide 12 (PAMP12) than to cortistatin; thus, this receptor was suggested to act physiologically as a PAMP receptor in the adrenal gland rather than as a cortistatin receptor (Kamohara *et al.* 2005). It has also been demonstrated that in human tissues cortistatin, but not somatostatin, binds to the ghrelin/GH secretagog receptor (GHS-R), suggesting a possible functional interaction between cortistatin and the ghrelin/GHS-R system (Spier & de Lecea 2000, Deghenghi *et al.* 2001b, Muccioli *et al.* 2001).

An inhibitory (dose-related) action of cortistatin on basal GH secretion similar to that of somatostatin has been demonstrated in man and rats *in vivo* (Deghenghi *et al.* 2001a, Broglio *et al.* 2002a,b, Gottero *et al.* 2004). Moreover, cortistatin, like somatostatin, drastically attenuated the GH response to GHRH in healthy normal individuals (Broglio *et al.* 2002a,b, Gottero *et al.* 2004). In the pig, as in other mammalian species, somatostatin inhibits *in vitro* GH-releasing hormone (GHRH)-stimulated GH release at high doses (nM). However, low (pM) doses of somatostatin stimulate pig GH release from cultured somatotropes (Castaño *et al.* 1996, Ramírez *et al.* 1997a,b, Ramírez *et al.* 2002). The stimulatory action of somatostatin on GH release is mediated by stimulation of cAMP and nitric oxide (NO) production (Ramírez *et al.* 2002, Luque *et al.* 2005).

Given the unique action of somatostatin on GH release in pig pituitary cell cultures, we compared the effects of cortistatin with those of somatostatin on basal GH secretion. In light of the ability of cortistatin, but not somatostatin, to bind the GHS-R (Spier & de Lecea 2000, Deghenghi *et al.* 2001b, Muccioli *et al.* 2001), we also sought to determine whether cortistatin interacts with the regulation of GH release by ghrelin/GHS-R. To this end, we used a short form of cortistatin, cortistatin-8 (CST8), which lacks the sst-binding capacity of full-length cortistatin, but retains its GHS-R binding capacity, and may antagonize certain ghrelin actions (Sibilia *et al.* 2006).

Materials and methods

Reagents

Pig GH (pGH; USDA-B-1, AFP-11716C) was kindly supplied by Dr A F Parlow, from the Pituitary Hormones and Antisera Center, Harbor-University of California-Los Angeles Medical Center. D-Val-modified minimum essential medium (MEM), Hepes, collagenase type V, trypsin type I, soybean trypsin inhibitor I, DNase I, gentamicin, antibiotic-antimycotic solution, BSA and all other reagents were purchased from Sigma,

unless otherwise specified. Fetal bovine serum (FBS) was obtained from Sera-Lab (Crawley Down, UK). Human cortistatin was used, as pig cortistatin has not yet been cloned, and was purchased from Peninsula (St Helens, UK). Somatostatin (1–14) was from Biogenesis (Poole, UK), GHRH (1–29) and anti-pGH were from UCB Bioproducts (Brain L'Alleud, Belgium) and human ghrelin was from Bachem (Merseyside, UK). Synthetic cortistatin-8 (CST8) was kindly provided by Dr Ezio Ghigo of the University of Turin (Turin, Italy) and the non-peptidyl GHS, L-163 255, by Merck. Tissue culture plastic ware was from Costar (Cambridge, MA, USA). Stock solutions of cortistatin, CST8, somatostatin and ghrelin were prepared with distilled deionized water, whereas GHRH was dissolved in ethanol. Aliquots of concentrated stock solutions were stored at -20°C until use, and then they were diluted to final concentrations in MEM.

Animals and tissues

Pituitary glands from female Large-White/Landrace pigs (4–6 months old) were obtained from a local abattoir. According to European Regulations for Animal Care, after electrical stunning, animals were killed by exsanguination and immediately decapitated. Pituitaries were immediately excised and stored in sterile cold (4°C) MEM supplemented with 0.3% BSA, 0.58% Hepes, 0.22% NaHCO_3 and 1% antibiotic-antimycotic solution (100 UI/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B). In the laboratory, pituitaries were washed twice with fresh medium, and the posterior lobes were discarded.

Pituitary cell dispersion and separation of subpopulations

Anterior pituitaries were dispersed into single cells by the enzymatic and mechanical method described previously (Torronteras *et al.* 1993, Castaño *et al.* 1996, Ramírez *et al.* 2002). For each experiment, three to four anterior pituitary glands were pooled and dispersed together, and the cell suspension obtained (initial cell suspension (ICS)) was filtered through a nylon gauze (100 µm mesh). The cell number was assessed in a hemocytometer, and cell viability, as determined by the Trypan blue exclusion test, was consistently over 85%. Initial cell suspension (ICS, $30\text{--}40 \times 10^6$ cells) was centrifuged (3000 g, 25 min) in a hyperbolic, continuous Percoll density gradient (Amersham, Buckinghamshire, UK) at 25–80% ($1033\text{--}1121\text{ g/cm}^3$) to separate the two subpopulations of pig somatotropes of low (LD; $1051\text{--}1064\text{ g/cm}^3$) and high (HD; $1076\text{--}1098\text{ g/cm}^3$) density, which have been characterized previously in our laboratory (Torronteras *et al.* 1993, Castaño *et al.* 1996, Ramírez *et al.* 2002).

Cell culture and experimental design

Monodispersed cells were plated onto 24-well culture plates at a density of 3×10^5 cells/well of ICS, LD and HD cells in 1 ml culture medium supplemented with 10% FBS and 0.1% gentamycin sulfate (50 µg/ml). After a 3-day culture period at 37 °C in a humidified atmosphere (95% air: 5% CO₂), the medium was removed and cells were preincubated in 1 ml serum-free MEM for 4 h to stabilize basal GH secretion. Then, cells were incubated for 30 min with the test substance or the corresponding control vehicle. Medium was then collected, centrifuged (2000 *g* for 5 min) and stored at -20 °C until GH determination.

Enzyme immunoassay

pGH concentration in the culture media was measured by a homologous enzyme immunoassay (EIA) procedure, as described in detail previously (Castaño *et al.* 1996), using a Rosys Anthos 2010 plate reader. Hormone employed in the EIA for both plate coating and standard was pGH, and the primary antiserum was a specific anti-pGH (developed in rabbit) at a dilution of 1:200 000. Sensitivity of the EIA was 0.65 ± 0.12 ng of pGH/well. A minimum of four replicate wells was employed to test the effect of each agent.

RNA extraction and multiplex RT-PCR of pig pituitary receptors

Given the distinct ability of cortistatin, but not somatostatin to bind to the human GHS-R (Deghenghi *et al.* 2001*b*, Muccioli *et al.* 2001), we sought to determine whether the differential GH response to these peptides could be associated with a differential expression of GHS-R as well as GHRH receptor (GHRH-R). To this end, total pituitary RNA was extracted from ICS-, LD- and HD-cultured cells and reverse transcribed, and receptor mRNA levels were determined by semiquantitative multiplex PCR, as previously developed and validated in our laboratory (Luque *et al.* 2004*a,b*). Hypoxanthine phosphoribosyl-transferase (HPRT) was used as an endogenous control.

Statistical analyses

A minimum of three or four replicate wells per treatment was tested in each experiment. Samples from each experiment were analyzed in the same assay and expressed as a percentage of the corresponding control value. Results are presented as mean $\pm$ S.E.M. of at least three experiments performed on different pituitary cell preparations. Statistical analysis was carried out by one-way ANOVA, followed by a statistical test for multiple comparisons (Duncan's multiple-range test and

critical ranges) by use of the software package Statistica (StatSoft, Tulsa, OK, USA). Differences were considered significant at $P < 0.05$.

Results

Effect of cortistatin and somatostatin on pGH release

Incubation of cultures of porcine pituitary cells (ICS) with increasing doses of somatostatin (SRIF) confirmed and extended our previous observations by showing that a broad range of low SRIF doses (10^{-18} to 10^{-10} M) stimulate GH release, whereas high, micromolar doses of SRIF (10^{-8} and 10^{-6} M) do not affect basal GH secretion (Fig. 1A).

To analyze the dose-dependent effect of cortistatin, porcine pituitary cells (ICS) were incubated with a wide range of concentrations of this peptide (10^{-20} – 10^{-6} M). As illustrated in Fig. 1B, cortistatin stimulated GH secretion in a dose-dependent manner. In particular, 10^{-18} , 10^{-16} and 10^{-14} M doses of cortistatin significantly increased GH secretion compared with vehicle-treated control.

Interaction of cortistatin with hypothalamic peptides, GHRH and SRIF

As shown in Fig. 2A, a high dose of cortistatin (10^{-7} M) completely abolished GHRH-induced GH release. Conversely, a low dose of cortistatin (10^{-15} M) stimulated GH secretion without modifying the stimulatory effect of GHRH.

Cortistatin and SRIF at a low dose (10^{-15} M) induced comparable increases in GH release (Fig. 2B). Combined administration of low doses of SRIF and cortistatin increased GH release to the same level as that observed when each peptide was applied separately.

Effect of cortistatin on pig somatotrope subpopulations

Similar to the stimulatory effect observed with low-dose cortistatin in whole cultures of pig pituitary cells (ICS), a low dose (10^{-15} M) of cortistatin stimulated GH release in both LD (Fig. 3A) and HD (Fig. 3B) subpopulations of porcine somatotropes. However, a high dose (10^{-7} M) of cortistatin stimulated GH release only in HD somatotropes (Fig. 3B). In contrast, a high dose (10^{-7} M) of cortistatin blocked the GH stimulatory effect of GHRH in LD somatotropes (Fig. 3A), but this effect was not observed in HD somatotropes (Fig. 3B).

Quantification of mRNA levels of GHRH-R and GHS-R

Examination of the relative expression levels of GHRH-R and GHS-R in somatotrope subpopulations

Figure 1 Dose–response effects of somatostatin (A) and cortistatin (B) on pig GH release *in vitro*. Cultures were incubated with MEM (0) or 10^{-20} M to 10^{-6} M somatostatin or cortistatin for 30 min at 37 °C. Data are expressed as a percentage of basal values (100%) in control experiments, and are the mean $\pm$ S.E.M. of five (somatostatin) and nine (cortistatin) separate experiments. Values that do not share a common letter are statistically different $P < 0.05$. CST, cortistatin; SRIF, somatostatin.

revealed that both receptors were more abundantly expressed in HD cells than in LD or ICS cells (Fig. 4).

Effect of CST8 on GH release

To compare the effects of CST8 with those of SRIF and cortistatin, total pituitary cell cultures (ICS) were treated with a wide range of CST8 doses (Fig. 5). In these assays, only a single low dose (10^{-15} M) of this peptide significantly stimulated GH release to 162% of control

Figure 2 Secretory response of cultured pig pituitary cells to treatment with two doses of cortistatin (10^{-7} and 10^{-15} M) $\pm$ 10^{-8} M GHRH (A) or combined low doses (10^{-15} M) of somatostatin and cortistatin (CST) (B). Data are expressed as a percentage of basal values (100%) in control experiments, and are the mean $\pm$ S.E.M. of four separate experiments. $P < 0.05$: a, vs control; b, vs GHRH alone.

levels. In two additional experiments, CST8 at 10^{-16} and 10^{-14} M also stimulated GH release significantly, albeit moderately, to 132% and 142% of vehicle-treated control respectively (data not shown).

Interaction of CST8 with GHRH, full-length cortistatin, ghrelin and the nonpeptidyl GHS agonist, L-163 255

As shown in Fig. 6A, a high dose (10^{-7} M) of CST8 did not inhibit GHRH-stimulated GH release. Interestingly, a low dose (10^{-15} M) of CST8 not only increased GH release, but also acted additively with GHRH to further stimulate GH secretion. On the other hand, the stimulatory effect of a low dose (10^{-15} M) of CST8 was blocked by a high dose (10^{-7} M) of cortistatin (Fig. 6B).

Figure 3 Secretory response of cultured pig pituitary cells to treatment with two doses of cortistatin (10^{-7} and 10^{-15} M) $\pm$ 10^{-8} M GHRH in LD (A) and HD (B) pig pituitary subpopulation. Data are expressed as a percentage of basal values (100%) in control experiments, and are the mean $\pm$ S.E.M. of four separate experiments. $P < 0.05$: a, vs control; b, vs GHRH alone.

Conversely, a high dose (10^{-7} M) of CST8 did not inhibit GH release induced by low-dose (10^{-15} M) cortistatin.

We next investigated the possible interaction of CST8 with ghrelin and a nonpeptidyl GHS agonist, L-163 255. As shown in Fig. 7A, high-dose (10^{-7} M) CST8 did not inhibit ghrelin-induced GH release. Interestingly, combined administration of both high and low doses of CST8 (10^{-7} and 10^{-15} M respectively) with ghrelin induced an additive stimulation of GH release. In contrast, high-dose (10^{-7} M) CST8 blocked the stimulatory effect of L-163 255 on GH release (Fig. 7B) while low-dose (10^{-15} M) CST8 did not alter L-163 255-stimulated GH secretion.

Figure 4 Expression levels of pig pituitary GHRH-R and GHS-R in ICS, LD and HD cells using multiplex RT-PCR. Data are expressed as a percentage of ICS cell expression levels (100%), and are the mean $\pm$ S.E.M. of three separate experiments. $P < 0.05$: a, vs ICS.

Figure 5 Dose-response effects of CST8 on pig GH release *in vitro*. Cultures were incubated with MEM (control) or 10^{-21} M to 10^{-7} M CST8 for 30 min at 37 °C. Data are expressed as percentage of basal values (100%) in control experiments, and are the mean $\pm$ S.E.M. of six separate experiments. $P < 0.05$: a, vs control.

Discussion

Somatostatin is commonly regarded as an inhibitor of GH release in rodents and man (Patel 1999). However, our results and those described by other groups indicate that somatostatin can stimulate GH release *in vitro* (Castaño *et al.* 1996, Torronteras *et al.* 1996, 1997,

Figure 6 Secretory response of cultured pig pituitary cells to treatment with two doses of CST8 (10^{-7} and 10^{-15} M) $\pm 10^{-8}$ M GHRH (A) or two doses (10^{-7} and 10^{-15} M) of full-length cortistatin (B). Data are expressed as percentage of basal values (100%) in control experiments, and are the mean $\pm$ S.E.M. of five separate experiments. $P < 0.05$: a, vs control; b, vs 10^{-15} M CST8; c, vs GHRH.

Ramírez *et al.* 1997a), can stimulate or enhance GH secretion *in vivo* in pigs (Anderson *et al.* 1991, Farmer *et al.* 1992), and can also enhance GH secretion *in vivo* in other species (Turner & Tannenbaum 1995, Leal-Cerro *et al.* 2002). In this report, we demonstrate that somatostatin at a wide range of low doses (10^{-18} – 10^{-10} M) directly stimulates GH release in pig pituitary cells, in a dose-dependent manner. At higher doses (10^{-8} M or greater), somatostatin had no effect on basal GH secretion. Interestingly, cortistatin, like somatostatin,

Figure 7 Secretory response of cultured pig pituitary cells to treatment with two doses of CST8 (10^{-7} and 10^{-15} M) in combination with 10^{-8} M ghrelin (A) or 10^{-8} M L-163 255 (B). Data are expressed as percentage of basal values (100%) in control experiments, and are the mean $\pm$ S.E.M. of five (ghrelin) and four (L-163 255) separate experiments. $P < 0.05$: a, vs control; b, vs ghrelin or L-163 255 alone; c, vs 10^{-8} M CST8; d, vs 10^{-15} M CST8.

also stimulated GH release from cultured porcine somatotropes when applied at low doses, albeit the magnitude and dose-dependency of its effects were less patent.

We also investigated the possible interaction of cortistatin and GHRH in somatotrope cells. Our group has previously shown that a high dose of somatostatin inhibited GHRH-induced GH release (via inhibition of adenylate cyclase (AC)), whereas a low dose did not alter the effect of GHRH (Castaño *et al.* 1996, Ramírez *et al.* 1997a). When pig pituitary cells were treated with a combination of cortistatin and GHRH, high doses of cortistatin (10^{-7} M) fully abolished GHRH-induced GH release, whereas a low dose (10^{-15} M) of cortistatin

stimulated GH secretion without altering the effect of GHRH. Therefore, these data demonstrate that the effects of cortistatin in pig are identical to those of somatostatin, suggesting that the actions of these peptides are exerted through the same receptors and/or intracellular signaling mechanism (Ramírez *et al.* 2002, Luque *et al.* 2005, 2006). This hypothesis is supported by the observation that combined administration of a low, stimulatory dose of both somatostatin and cortistatin did not augment GH secretion over that achieved by each peptide alone, while a high dose of either peptide suppressed the stimulation caused by a low dose of the other peptide (data not shown).

We have previously reported that the population of pituitary somatotropes is in fact composed of two morphologically and functionally distinct cell subpopulations (LD and HD) that can be isolated by Percoll density gradient (Castaño *et al.* 1996, Ramírez *et al.* 1997b, 2002). In LD cells, the inhibitory effect of high-dose somatostatin on GHRH-stimulated GH release and the stimulatory effect of low-dose somatostatin on basal GH release can be observed. However, in the HD subpopulation, somatostatin stimulates GH release irrespective of dose and has no effect on GHRH-stimulated GH release (Ramírez *et al.* 1997b). Therefore, we studied the effect of cortistatin on GH release in LD and HD subpopulations, and our results demonstrate that cortistatin evokes the same GH response as that shown previously for somatostatin. These results underscore the functional similarities between cortistatin and somatostatin in the control of GH release, and strongly support the view that their actions in somatotropes are exerted through the same receptors. Recent studies from our group using nonpeptidyl somatostatin agonists, selective for each of the five sst, suggest that sst1 and sst2 mediate the inhibitory effects of somatostatin on GH release, whereas sst5 activation exerts stimulatory effects on GH release (Luque *et al.* 2006). In line with this, recent results indicate that sst5 is expressed at higher levels in HD subpopulations, where somatostatin and cortistatin at both high and low doses have a stimulatory effect (Delgado *et al.* 2002, Luque *et al.* 2006).

We have demonstrated that the actions of cortistatin are virtually identical to those observed with somatostatin on pig somatotropes and therefore their actions might be exerted via the same sst receptors. However, given the ability of cortistatin, but not somatostatin, to bind the human GHS-R (Deghenghi *et al.* 2001b,c, 2003, Muccioli *et al.* 2001), it has been suggested that some actions of cortistatin could be conveyed through GHS-R signaling (Spier & de Lecea 2000, Muccioli *et al.* 2001, Deghenghi *et al.* 2001a,b,c, 2003). In line with this, examination of relative expression levels of GHS-R in the somatotrope cell subtypes reveals that HD cells, which are responsive only to the stimulatory actions of

somatostatin and cortistatin, express more GHS-R than the LD subpopulation. These observations would suggest that the stimulatory actions on pig GH release of cortistatin (and perhaps somatostatin) could be mediated, at least in part, via GHS-R. To explore this possibility, pig pituitary cells were treated with a truncated cortistatin analog, CST8, which is able to bind selectively only the GHS-R, and not the sst. In fact, CST8 has recently been shown to behave as an antagonist of GHS-R by counteracting the response of ghrelin on gastric acid secretion (Sibilia *et al.* 2006). In our model, however, CST8 stimulated GH release, although, unlike intact cortistatin or somatostatin, it elicited a more restricted effect, in that it acted only at doses from 10^{-16} to 10^{-14} M. Consistent with the inability of CST8 to bind to sst, a high dose of CST8 did not inhibit GHRH-stimulated GH release. Interestingly, a low dose of CST8 (10^{-15} M) additively augmented GHRH-induced GH release, thus showing that CST8 exerts its effects, at least in part, through different receptor(s) and signaling mechanisms than those used by full-length cortistatin. Consistent with this idea, the stimulatory effect of low doses (10^{-15} M) of CST8 was blocked by 10^{-7} M full-length cortistatin, whereas a high dose (10^{-7} M) of CST8 did not inhibit GH release induced by low-dose cortistatin.

In an attempt to clarify whether CST8 exerts its actions on pig cells by binding to the GHS-R, as previously reported in rats (Sibilia *et al.* 2006), we tested the effects of CST8 in combination with ghrelin. Surprisingly, a high dose of CST8 (10^{-7} M) did not inhibit GH release induced by ghrelin. In fact, high and low doses of CST8 (10^{-7} and 10^{-15} M) combined with 10^{-8} M ghrelin induced an additive stimulation of GH release. These results differ from the recently described antagonistic action of CST8 on ghrelin inhibition of gastric acid secretion when administered centrally in rats *in vivo*, which was attributed to CST8 binding to GHS-R1a (Sibilia *et al.* 2006). This apparent discrepancy in CST8 action can relate to the different species, doses and biologic model studied (ghrelin i.c.v. action on rat neurons vs *in vitro* ghrelin action on pig somatotropes). However, our present data strongly indicate that CST8 activates a receptor and/or signaling pathway distinct from that used by ghrelin in pig somatotropes, or, as recently proposed for ghrelin and synthetic GHSs (Holst *et al.* 2005), CST8 may be acting via the same GHS-R as ghrelin, but by promoting additional, distinct receptor–receptor interactions (dimers) that would thereby enhance the GH-releasing action of ghrelin. In agreement with this latter idea, we found that the combined administration of a low dose of CST8 with 10^{-8} M of the nonpeptidyl GHS agonist, L-163 255, did not cause an additive stimulatory effect comparable to that found for ghrelin. Furthermore, 10^{-8} M CST8 inhibited L-163 255-stimulated GH release, thereby

Figure 8 Working model proposed to explain the effect of somatostatin, cortistatin, CST8, ghrelin and nonpeptidyl GHSs (L-163 255) on pig somatotropes *in vitro* (see text for details).

suggesting that CST8 antagonizes a receptor subtype or receptor conformation specifically activated by this synthetic, nonpeptidyl GHS. Interestingly, we have previously reported that, while ghrelin stimulates GH release in pig somatotropes by activating both AC and phospholipase C/protein kinase C (PLC/PKC) second messenger routes (Malagón *et al.* 2003), L-163 255 activates only PLC/PKC (Gracia-Navarro *et al.* 2002). In this context, our results suggest that CST8 modulates PLC/PKC signaling via a receptor distinct from that used by ghrelin or by enabling conformations of the known GHS-R (dimers) distinct from those affected by ghrelin (Holst *et al.* 2005).

To summarize our present results in the context of previous findings, we propose a working model (Fig. 8) wherein somatostatin and full-length cortistatin bind to sst1 and sst2 to exert their inhibitory actions on GH release in pig somatotropes by inhibition of AC pathway, whereas sst5 mediate their stimulatory actions (Luque *et al.* 2006) probably via AC activation (Ramírez *et al.* 2002). Moreover, ghrelin stimulates GH release by activating both AC and PLC pathways (Malagón *et al.* 2003), either by binding to distinct binding pockets of the GHS-R (Feighner *et al.* 1998) and/or enabling dimeric GHS-R forms (Holst *et al.* 2005), or by binding and activating an alternate, different receptor (Chen 2000). In this working model, we also propose that L-163 255 can bind only to the specific GHS-R-binding pocket or receptor combination that allows coupling to the PLC/PKC route to stimulate pig GH release (Gracia-Navarro *et al.* 2002), and that CST8 can use a similar binding mechanism to activate or impair these signals, depending on the presence of other GHS-R agonists. Studies in our laboratory are continuing to clarify these complex signaling pathways.

In conclusion, our results demonstrate that cortistatin strongly mimics the dual *in vitro* actions of somatostatin on GH pig release, suggesting that both peptides mediate their endocrine effects on somatotropes through the same receptors and signaling mechanisms. When the present findings *in vitro* are viewed together with previous reports *in vivo* on the endocrine actions of cortistatin (Deghenghi *et al.* 2001a, Broglio *et al.* 2002a,b, Gottero *et al.* 2004) and with the recent demonstration of immunoreactive cortistatin in normal and tumoral human neuroendocrine tissues, which express high levels of sst (Allia *et al.* 2005), it becomes apparent that the possible local, auto/paracrine actions of cortistatin in pituitary and other endocrine tissues merit further investigation.

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References

- Allia E, Tarabra E, Volante M, Cerrato M, Ghigo E, Muccioli G & Papotti M 2005 Expression of cortistatin and MrgX2, a specific cortistatin receptor, in human neuroendocrine tissues and related tumours. *Journal of Pathology* **207** 336–345.
- Anderson LL, Ford JJ, Klindt J, Molina JR, Vale WW & Rivier J 1991 Growth hormone and prolactin secretion in hypophyseal stalk-transected pigs as affected by growth hormone and prolactin-releasing and inhibiting factors. *Proceedings of the Society for Experimental Biology and Medicine* **196** 194–202.
- Broggio F, Arvat E, Benso A, Gottero C, Prodham F, Grottoli S, Papotti M, Muccioli G, van der Lely AJ, Deghenghi R & Ghigo E 2002a Endocrine activities of cortistatin-14 and its interaction with GHRH and ghrelin in humans. *Journal of Clinical Endocrinology and Metabolism* **87** 3783–3790.
- Broggio F, Koetsveld PVP, Benso A, Gottero C, Prodham F, Papotti M, Muccioli G, Gauna C, Hoffland L, Deghenghi R, Arvat E, Van Der Lely AJ & Ghigo E 2002b Ghrelin secretion is inhibited by either somatostatin or cortistatin in humans. *Journal of Clinical Endocrinology and Metabolism* **87** 4829–4832.
- Castaño JP, Torronteras R, Ramirez JL, Gribouval A, Sanchez-Hormigo A, Ruiz-Navarro A & Gracia-Navarro F 1996 Somatostatin increases growth hormone (GH) secretion in a subpopulation of porcine somatotropes: evidence for functional and morphological heterogeneity among porcine GH-producing cells. *Endocrinology* **137** 129–136.
- Chen C 2000 Growth hormone secretagogue actions on the pituitary gland: multiple receptors for multiple ligands? *Clinical and Experimental Pharmacology and Physiology* **27** 323–329.
- Dalm VA, Van Hagen PM, de Krijger RR, Kros JM, Van Koetsveld PM, Van Der Lely AJ, Lamberts SW & Hoffland LJ 2004 Distribution pattern of somatostatin and cortistatin mRNA in human central and peripheral tissues. *Clinical Endocrinology* **60** 625–629.
- Deghenghi R, Avallone R, Torsello A, Muccioli G, Ghigo E & Locatelli V 2001a Growth hormone-inhibiting activity of cortistatin in the rat. *Journal of Endocrinological Investigation* **24** RC31–33.
- Deghenghi R, Papotti M, Ghigo E & Muccioli G 2001b Cortistatin, but not somatostatin, binds to growth hormone secretagogue (GHS) receptors of human pituitary gland. *Journal of Endocrinological Investigation* **24** RC1–3.
- Deghenghi R, Papotti M, Ghigo E, Muccioli G & Locatelli V 2001c Somatostatin octapeptides (lanreotide, octreotide, vapreotide, and their analogs) share the growth hormone-releasing peptide receptor in the human pituitary gland. *Endocrine* **14** 29–33.
- Deghenghi R, Broggio F, Papotti M, Muccioli G & Ghigo E 2003 Targeting the ghrelin receptor: orally active GHS and cortistatin analogs. *Endocrine* **22** 13–18.
- de Lecea L 2005 Cortistatin: a natural somatostatin analogue. *Somatostatin and Its Natural and Synthetic Analogues*. Turin, Italy. Abstract.
- de Lecea L, Criado JR, Próspero-García O, Gautvik KM, Schweitzer P, Danielson PE, Dunlop CL, Siggins GR, Henriksen SJ & Sutcliffe JG 1996 A cortical neuropeptide with neuronal depressant and sleep-modulating properties. *Nature* **381** 242–245.
- de Lecea L, Ruiz-Lozano P, Danielson PE, Pelle-Kirley J, Foye PE, Frankel WN & Sutcliffe JG 1997a Cloning, mRNA expression, and chromosomal mapping of mouse and human preprocortistatin. *Genomics* **42** 499–506.
- de Lecea L, del Río JA, Criado JR, Alcántara S, Morales M, Danielson PE, Henriksen SJ, Soriano E & Sutcliffe JG 1997b Cortistatin is expressed in a distinct subset of cortical interneurons. *Journal of Neuroscience* **17** 5868–5880.
- Delgado E, Castaño JP, Sánchez-Hormigo A, Luque RM, Peinado JR, Garrido JJ, Gracia-Navarro F & Malagón MM 2002 Molecular characterization of pig somatostatin receptors. 21st Conference of European Comparative Endocrinologists. Bonn, Germany. Abstract.
- Farmer C, Randall G & Brazeau P 1992 *In vivo* growth hormone (GH) response to human GH-releasing factor (GRF) or somatostatin (SRIF) in foetal pigs. *Journal of Developmental Physiology* **17** 93–97.
- Feighner SD, Howard AD, Prendergast K, Palyha OC, Hreniuk DL, Nargund R, Underwood D, Tata JR, Dean DC, Tan CP, McKee KK, Woods JW, Patchett AA, Smith RG & Van der Ploeg LH 1998 Structural requirements for the activation of the human growth hormone secretagogue receptor by peptide and nonpeptide secretagogues. *Molecular Endocrinology* **12** 137–145.
- Fukusumi S, Kitada C, Takekawa S, Kizawa H, Sakamoto J, Miyamoto M, Hinuma S, Kitano K & Fujino M 1997 Identification and characterization of a novel human cortistatin-like peptide. *Biochemical and Biophysical Research Communications* **232** 157–163.
- Gottero C, Prodham F, Destefanis S, Benso A, Gauna C, Me E, Filtri L, Riganti F, Van Der Lely AJ, Ghigo E & Broggio F 2004 Cortistatin-17 and -14 exert the same endocrine activities as somatostatin in humans. *Growth Hormone and IGF Research* **14** 382–387.
- Gracia-Navarro F, Castaño JP, Malagón MM, Sanchez-Hormigo A, Luque RM, Hickey GJ, Peinado JR, Delgado E & Martinez-Fuentes AJ 2002 Research progress in the stimulatory inputs regulating growth hormone (GH) secretion. *Comparative Biochemistry and Physiology. Biochemistry and Molecular Biology* **132** 141–150.
- Holst B, Brandt E, Bach A, Heding A & Schwartz TW 2005 Nonpeptide and peptide growth hormone secretagogues act both as ghrelin receptor agonist and as positive or negative allosteric modulators of ghrelin signaling. *Molecular Endocrinology* **19** 2400–2411.
- Kamohara M, Matsuo A, Takasaki J, Kohda M, Matsumoto M, Matsumoto S, Soga T, Hiyama H, Kobori M & Katou M 2005 Identification of MrgX2 as a human G-protein-coupled receptor for proadrenomedullin N-terminal peptides. *Biochemical Biophysical Research Communications* **330** 1146–1152.
- Leal-Cerro A, Soto A, Martínez MA, Alvarez P, Isidro L, Casanueva FF, Diéguez C & Cordido F 2002 Effect of withdrawal of somatostatin plus growth hormone (GH)-releasing hormone as a stimulus of GH secretion in Cushing's syndrome. *Clinical Endocrinology (Oxford)* **57** 745–749.
- Luque RM, Park S, Peng XD, Delgado E, Gracia-Navarro F, Kineman RD, Malagón MM & Castaño JP 2004a Homologous and heterologous in vitro regulation of pig pituitary somatostatin receptor subtypes, sst1, sst2 and sst5 mRNA. *Journal of Molecular Endocrinology* **32** 437–448.
- Luque RM, Kineman RD, Park S, Peng XD, Gracia-Navarro F, Castaño JP & Malagón MM 2004b Homologous and heterologous regulation of pituitary receptors for ghrelin and growth hormone-releasing hormone. *Endocrinology* **145** 3182–3189.
- Luque RM, Rodríguez-Pacheco F, Tena-Sempere M, Gracia-Navarro F, Malagón MM & Castaño JP 2005 Differential contribution of nitric oxide and cGMP to the stimulatory effects of GHRH and low-dose somatostatin on growth hormone (GH) release from somatotropes. *Journal of Neuroendocrinology* **17** 577–582.
- Luque RM, Duran-Prado M, García-Navarro S, Gracia-Navarro F, Kineman RD, Malagón MM & Castaño JP 2006 Identification of the somatostatin receptor subtypes (sst) mediating the divergent, stimulatory/inhibitory actions of somatostatin on growth hormone secretion. *Endocrinology* In Press.
- Malagón MM, Luque RM, Ruiz-Guerrero E, Rodríguez-Pacheco F, Gracia-Navarro S, Casanueva FF, Gracia-Navarro F & Castaño JP 2003 Intracellular signaling mechanisms mediating ghrelin-stimulated growth hormone release in somatotropes. *Endocrinology* **144** 5372–5380.
- Muccioli G, Papotti M, Locatelli V, Ghigo E & Deghenghi R 2001 Binding of 125I-labeled ghrelin to membranes from human

- hypothalamus and pituitary gland. *Journal of Endocrinological Investigation* **24** RC7–9.
- Patel YC 1999 Somatostatin and its receptor family. *Frontiers in Neuroendocrinology* **20** 157–198.
- Ramírez JL, Castaño JP & Gracia-Navarro F 1997a Somatostatin at low doses stimulates growth hormone release from intact cultures of porcine pituitary cells. *Hormone and Metabolic Research* **30** 175–177.
- Ramírez JL, Torronteras R, Castaño JP, Sánchez-Hormigo A, Garrido JC, García-Navarro S & Gracia-Navarro F 1997b Somatostatin plays a dual, stimulatory/inhibitory role in the control of growth hormone secretion by two somatotrope subpopulations from porcine pituitary. *Journal of Neuroendocrinology* **9** 841–848.
- Ramírez JL, Gracia-Navarro F, García-Navarro S, Torronteras R, Malagón MM & Castaño JP 2002 Somatostatin stimulates GH secretion in two porcine somatotrope subpopulations through a cAMP-dependent pathway. *Endocrinology* **143** 889–897.
- Robas N, Mead E & Fidock M 2003 MrgX2 is a high potency cortistatin receptor expressed in dorsal root ganglion. *Journal of Biological Chemistry* **278** 44400–44404.
- Sánchez-Alavez M, Gomez-Chavarín M, Navarro L, Jiménez-Anguiano A, Murillo-Rodríguez E, Prado-Alcala RA, Drucker-Colin R & Próspero-García O 2000 Cortistatin modulates memory processes in rats. *Brain Research* **858** 78–83.
- Sibilia V, Muccioli G, Deghenghi R, Pagani F, De Luca V, Rapetti D, Locatelli V & Netti C 2006 Evidence for a role of the GHS-R1a receptors in ghrelin inhibition of gastric acid secretion in the rat. *Journal of Neuroendocrinology* **18** 122–128.
- Siehl S, Seuwen K & Hoyer D 1998 [125I]Tyr10-cortistatin14 labels all five somatostatin receptors. *Naunyn-Schmiedeberg's Archives of Pharmacology* **357** 483–489.
- Spier AD & de Lecea L 2000 Cortistatin: a member of the somatostatin neuropeptide family with distinct physiological functions. *Brain Research. Brain Research Reviews* **33** 228–241.
- Spier AD, Fabre V & de Lecea L 2005 Cortistatin radioligand binding in wild-type and somatostatin receptor-deficient mouse brain. *Regulatory Peptides* **124** 179–186.
- Torronteras R, Castaño JP, Ruiz-Navarro A & Gracia-Navarro F 1993 Application of a Percoll density gradient to separate and enrich porcine pituitary cell types. *Journal of Neuroendocrinology* **5** 257–266.
- Torronteras R, Gracia-Navarro F & Elsaesser F 1996 Different effects of somatostatin on *in vitro* growth hormone release in two porcine breeds with different growth rates. *Journal of Neuroendocrinology* **8** 891–900.
- Torronteras R, Gracia-Navarro F & Elsaesser F 1997 Control of growth hormone secretion from porcine fetal and neonatal pituitary tissue *in vitro* by growth hormone-releasing hormone, somatostatin and insulin-like growth factor. *Neuroendocrinology* **65** 117–128.
- Turner JP & Tannenbaum GS 1995 *In vivo* evidence of a positive role for somatostatin to optimize pulsatile growth hormone secretion. *American Journal of Physiology* **269** E683–690.

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