Homologous and heterologous *in vitro* regulation of pig pituitary somatostatin receptor subtypes, sst1, sst2 and sst5 mRNA

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Abstract

Somatostatin (SRIF) is commonly regarded as an inhibitor of GH release in rodents and humans. However, in pigs, SRIF can stimulate the release of GH at low (picomolar) doses, while inhibiting GHRH-stimulated GH release at high (nanomolar) doses in primary pituitary cell cultures. One possible mechanism by which pig cells respond differently to the actions of SRIF is by differential expression and regulation of SRIF receptor subtypes. As no information is available on the homologous regulation of SRIF receptors in pigs, we examined the acute (4 h) *in vitro* effects of SRIF on mRNA levels of SRIF receptors sst1, sst2 and sst5 by multiplex RT-PCR. These particular sst subtypes were selected because all three have been implicated in the inhibitory effects of SRIF on GH release in both rodents and humans. At a high dose (10^{-7} M), SRIF stimulated the expression of sst1, sst2 and sst5 in pig pituitary cell cultures. At a low dose (10^{-13} M), SRIF markedly increased sst1, without affecting sst2 or sst5. Given that our laboratory has shown SRIF at high and low doses stimulates cAMP production in a subpopulation of pig somatotropes, we sought to determine if this signaling pathway may be responsible for the stimulatory effect of SRIF on its own receptor expression. The receptor-independent cAMP activator forskolin elevated sst1 and sst2 mRNA levels but did not affect sst5 expression, suggesting the stimulatory actions of high- and low-dose SRIF on sst1 and high-dose SRIF on sst2 mRNA levels can be mediated by activation of cAMP, whereas the stimulatory effect of high-dose SRIF on sst5 mRNA is elicited by a cAMP-independent pathway. Interestingly, both GHRH (10^{-8} M) and ghrelin (10^{-6} M), which release GH in pig pituitary cell cultures via cAMP-dependent mechanisms, decreased sst5 without altering sst1 or sst2 mRNA levels. Since the actions of GHRH and ghrelin on sst expression markedly contrasted with that observed for SRIF and forskolin these results clearly indicate GHRH and ghrelin are regulating sst5 mRNA levels by a cAMP-independent signaling pathway. Taken together, our results demonstrate that expression of pig SRIF receptors is under a complex, receptor subtype-selective regulation, wherein the concerted actions of key regulators of somatotrope function would play divergent and dose-dependent effects.

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Introduction

Somatostatin (SRIF), as its name implies, was first recognized as a neuroendocrine inhibitor of pituitary growth hormone (GH; somatotropin). However, since its discovery SRIF has been shown to inhibit also the release of thyrotropin, and to mediate the function of all pituitary cell types

depending on the physiological/hormonal environment of the tissue donor (Patel 1999). Moreover, within the brain, SRIF acts as a classic neurotransmitter, while in the periphery SRIF produced in the gastrointestinal tract, kidney and lymphoid tissue acts as a paracrine and endocrine factor to mediate a wide array of functions (Reisine & Bell 1995, Meyerhof 1998, Patel 1999, Benali *et al.*

2000, Krantic 2000, Csaba & Dournaud 2001). The multiple actions of SRIF are believed to be transduced by the differential use of six G-protein coupled receptor subtypes (*sst1*, *sst2A*, *sst2B*, *sst3*, *sst4* and *sst5*) encoded by five separate genes (*sst1*–*5*), all of which have been cloned in the human, rat and mouse (Reisine & Bell 1995, Meyerhof 1998, Patel 1999, Baumeister & Meyerhof 2000, Benali *et al.* 2000, Kraus *et al.* 2000, Csaba & Dournaud 2001). All six SRIF receptor subtypes are expressed within the pituitary gland. However, analysis of SRIF receptor subtype protein and mRNA levels within the pituitaries of rats, mice and humans demonstrates that *sst2* and *sst5* are the dominant receptor isoforms (Kumar *et al.* 1997). Also at a functional level, *sst2* and *sst5* play a dominant role in inhibition of GH release where *sst2*- and *sst5*-specific receptor agonists are 1000-fold more effective in suppressing GH-releasing hormone (GHRH)-mediated GH release compared with *sst1*, *sst3* and *sst4* agonists (Shimon *et al.* 1997*a,b*, Rohrer *et al.* 1998). Nevertheless, other studies suggest an important role for *sst1* in the control of basal GH release (Kreienkamp *et al.* 1999).

In pigs, unlike other mammals studied to date, SRIF plays a dual role in controlling GH secretion from somatotropes (Castaño *et al.* 1996, Ramírez *et al.* 1997, 1998, 2002). Previous studies from our laboratory have shown that low (picomolar) doses of SRIF actually stimulate GH release from cultured porcine somatotropes, whereas higher (nanomolar) concentrations of the peptide inhibit GHRH-stimulated but not basal GH release (Ramírez *et al.* 1997, 1998, 2002). The stimulatory action of SRIF on GH release is believed to be mediated by stimulation of a cAMP intracellular signal transduction pathway, in that adenylyl cyclase inhibitors block this effect (Ramírez *et al.* 2002). Species-specific differences in SRIF actions could be due to differential expression and ligand-dependent regulation of the various SRIF receptor subtypes. There is solid evidence that SRIF can acutely and selectively regulate the function of each SRIF receptor subtype through ligand-dependent receptor desensitization, internalization and degradation (Meyerhof 1998, Patel 1999, Benali *et al.* 2000, Csaba & Dournaud 2001). However, SRIF can also regulate its own receptor synthesis and this homologous regulation can be modified by heterologous signals via cross-talk of intracellular signaling pathways (Reisine & Bell

1995, Meyerhof 1998, Patel 1999, Baumeister & Meyerhof 2000, Benali *et al.* 2000, Kraus *et al.* 2000, Csaba & Dournaud 2001). Regulation of SRIF receptor gene expression by homo- and heterologous signals has been studied in different tissues and cell lines by using diverse technical approaches, including Northern blotting, RT-PCR, and heterologous and homologous transient transfection systems using SRIF receptor promoter-driven reporter plasmids (Reisine & Bell 1995, Meyerhof 1998, Patel 1999, Baumeister & Meyerhof 2000, Benali *et al.* 2000, Kraus *et al.* 2000, Csaba & Dournaud 2001). These studies have revealed that a variety of signals, including SRIF, GHRH, glucocorticoids, cAMP-elevating agents, gonadal steroids and growth factors, differentially control the expression of SRIF receptors in the pituitary and in pituitary-derived cell lines (Bruno *et al.* 1994*a,b*, Reisine & Bell 1995, Xu *et al.* 1995, Señaris *et al.* 1996, Djordjijevic *et al.* 1998, Kimura *et al.* 1998, Meyerhof 1998, Patel 1999, Baumeister & Meyerhof 2000, Benali *et al.* 2000, Kraus *et al.* 2000, Park *et al.* 2000, 2002, Csaba & Dournaud 2001). Nevertheless, very little is known on the regulation of SRIF receptor expression in species other than human, rat, and mouse.

Therefore, in the present study we sought to better understand the differential effects of SRIF on pig pituitary GH release by investigating the acute regulation of SRIF receptor expression by specifically focusing on the three SRIF receptor subtypes previously shown to be critical in GH regulation in other mammalian species, *sst1*, *sst2* and *sst5*. However, of these SRIF receptor subtypes, only *sst2* has been cloned in the pig (Matsumoto *et al.* 1994). To circumvent this problem, we used primer sets based on homologous regions of the rat, mouse and human *sst1* and *sst5* genes, to PCR amplify and obtain partial sequence of the pig *sst1* and *sst5* coding regions. With the published (Matsumoto *et al.* 1994) and new sequence data we developed and validated a multiplex RT-PCR assay that enabled the simultaneous assessment of the mRNA levels for porcine *sst1*, *sst2* and *sst5*. Using this approach, we have studied the expression of these receptors in cultures of pig pituitary cells in response to treatment with SRIF, GHRH, ghrelin and forskolin, a receptor-independent activator of the cAMP pathway. The results obtained provide, for the first time, basic information on homologous

and heterologous regulation of the expression of pig SRIF receptors, and demonstrate distinct patterns of response of each receptor subtype to the same signals.

Materials and methods

Reagents

Unless otherwise indicated, chemical products were purchased from Sigma Chemical Co. (St Louis, MO, USA) and tissue culture reagents and molecular biology reagents were obtained from GIBCO-BRL (Grand Island, NY, USA).

Animals, cell dispersion and cell culture

Prepubertal (4–6 month) female Large-White/Landrace pigs were killed according to the principles and procedures outlined in the NIH Guide for the Care and Use of Laboratory Animals of the University of Illinois (Chicago, IL, USA). After killing, pituitaries were immediately excised and stored (<15 min postmortem) in sterile cold (4 °C) culture medium (Minimum Essential Medium; MEM) containing 0.1% BSA, 100 µg/ml streptomycin and 100 U/ml penicillin. At the laboratory, posterior lobes were removed and the anterior pituitaries were dispersed following an enzymatic and mechanical dispersion protocol described in detail previously (Aleppo *et al.* 1997). Cells were plated onto 12-well tissue culture plates at a density of 800 000 cells/well in 1 ml α MEM supplemented with 0.1% BSA and antibiotics supplemented with 10% fetal bovine serum. After 3 days of culture, medium was removed and cells were preincubated with fresh, serum-free medium for 1 h at 37 °C. Medium was then replaced with fresh media containing the test substances at the appropriate concentrations or the corresponding control vehicle, and incubated for an additional 4 h at 37 °C.

RNA isolation

Total RNA extraction was carried out using the Tri Reagent protocol (Molecular Research Center, Cincinnati, OH, USA). To increase the efficiency of the reverse transcriptase (RT) reaction the aqueous phase was further purified by extraction

with phenol:chloroform:isoamyl alcohol (25:24:1; pH 5.2; Fisher Scientific, Pittsburgh, PA, USA). Following extraction the RNA was precipitated with isopropanol and the pellet washed with 70% ethanol, air dried, and resuspended in 10 mM Tris-HCl, pH 7.6, 1 mM EDTA buffer. Concentration and purity of the RNA samples were estimated by UV spectroscopy at 260 and 280 nm, and integrity confirmed by electrophoresis through 1% agarose gels stained with ethidium bromide.

Partial sequencing of pig *sst1* and *sst5* and PCR primer design for *sst1*, *sst2* and *sst5*

Of the three porcine SRIF receptor subtypes analyzed in the present study, only pig *sst2* had been previously cloned (Matsumoto *et al.* 1994). To obtain partial nucleotide sequences of pig *sst1* and *sst5* coding sequences that would enable the development of a quantitative RT-PCR, we designed PCR primer sets within highly homologous areas of the human, rat and mouse *sst1* and *sst5* coding sequences. Using these primers we PCR amplified cDNA obtained from pig pituitary RNA where product sizes and subsequent automated 3' and 5' sequencing (ABI PRISM 3100 Genetic Analyzer; Applied Biosystems, Foster City, CA, USA) demonstrated close similarity to the corresponding sequences in the homologous mammalian *sst1* and *sst5* receptors cDNA. Specifically, a fragment of 322 bp was obtained for pig *sst1* (GenBank Accession No. AY138806) that encompasses from the 5' end of transmembrane (TM) region 3 through the 5' end of intracellular loop 3. For pig *sst5*, we obtained a fragment of 201 bp (GenBank Accession No. AY138807), comprising the 5' end of intracellular loop 2 to the internal region of TM domain 5. These sequences were then used to design internal, pig-specific primers, which were selected on the basis of providing comparable annealing temperatures and compatible product size. Likewise, primers were designed for *sst2* using the known sequence (Matsumoto *et al.* 1994; GenBank Accession No. D21338), and the specificity of the fragment amplified was confirmed by sequencing. The coding sequences of the SRIF subtype receptors amplified were: (i) *sst1*, from third to fifth TM region (TM3–TM5), (ii) *sst2*, from fifth to seventh TM region (TM5–TM7), and (iii) *sst5*, from fourth to fifth TM region (TM4–TM5). Finally, porcine hypoxanthine

Table 1 Sequence of primers employed for the multiplex RT-PCR assay of SRIF receptors with corresponding size of the fragments amplified and their GenBank Accession Numbers

	Primer sequences (5'→3')	Size (bp)	GenBank Accession Number
Receptor			
<i>sst1</i>	Sense: CACCAGCATCTACTGTCTTAC Antisense: AAAGTGTACAACACGAAGC	258	AY138806
<i>sst2</i>	Sense: TTCATTATCTACGCCTTCATCC Antisense: GATTGTCCCTGCTTACTGTAC	422	D21338
<i>sst5</i>	Sense: TGCACCCCCCTTCGCTCTG Antisense: CCAGCTGAGGTTGCAGGTGT	149	AY138807
HPRT	Sense: TGAACGTCTTGCTCGAGATGTG Antisense: TTATATCGCCCGTTGACTGGTC	206	U69731

phosphoribosyltransferase (HPRT; GenBank Accession No. U69731) was selected as internal control and the appropriate primers were designed and tested, including the sequencing of the fragment amplified. (See Table 1 for specific primer sequences and PCR product sizes.)

Multiplex RT-PCR of pituitary *sst* mRNA

mRNA levels of *sst1*, *sst2* and *sst5* were assessed using semi-quantitative RT-PCR. First-strand cDNA for PCR was synthesized from 1 µg total RNA by reverse transcription using random hexamer primers according to the Superscript Preamplification System for First Strand Synthesis (Life Technologies, Inc., St Louis, MO, USA). All PCR reactions were performed under the following conditions: an initial denaturing step of 10 min at 95 °C followed by 27 cycles of 1 min denaturation at 95 °C, 1 min annealing at 61 °C, and 1 min extension at 72 °C with a 10 min final extension at 72 °C. PCR products derived from RNA were verified by their absence in reactions performed without RT.

The PCR reactions were performed in a 50 µl volume containing 2 µl RT reaction, 2 U Taq Gold polymerase (Perkin-Elmer Corp., Branchburg, NJ, USA), 1 × PCR buffer, 0.2 mM deoxy-NTPs, 1.5 mM MgCl₂ and 5 µCi [α -³²P]deoxy-CTP (800 Ci/mmol). The appropriate primer concentrations, 0.6 µM *sst1* primers, 0.3 µM *sst2* primers, 0.6 µM *sst5* primers and 0.04 µM HPRT primers, were empirically determined to achieve a final signal that was comparable for all PCR products within each reaction and that would provide non-competitive

and specific amplification for each PCR product. Consequently, this technique can only be used to compare the expression level of a single receptor type between experimental groups and not the relative expression levels between the various receptor subtypes.

PCR products (20 µl) were separated on 5% polyacrylamide gel. Gels were dried on chromatography paper and exposed to a phosphorimage screen. The intensity of each band was visualized and analyzed using image analysis software (Molecular Dynamics, Inc., Sunnyvale, CA, USA), where band intensity is expressed in pixels. In order to control for variability in the amount of total RNA used in the RT reaction and the efficiency of conversion of RNA to cDNA, SRIF receptor subtype mRNA levels, within the same reaction, were normalized to the HPRT mRNA and expressed as a percentage of the vehicle-treated levels (100%).

Statistical analysis

Data are expressed as means ± S.E.M. from three independent experiments performed on different pituitary cell preparations. In each experiment, treatment groups were tested in triplicate. All comparisons were made between samples electrophoresed on the same gel. Statistical analysis was carried out using a 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, Inc., Tulsa, OK, USA). Differences were considered significant at $P < 0.01$.

Results

Validation of the multiplex RT-PCR for quantification of mRNA levels of pig *sst* subtypes

Application of an RT-PCR technique provides a rapid, reliable and sensitive means to measure mRNA levels of several gene products in the same sample. However, because of the exponential nature of the PCR, accuracy of this method is critically dependent on several variables, including PCR cycle number and the amount of starting mRNA of the sample (Zamorano *et al.* 1996, Aleppo *et al.* 1997). Hence, these parameters must be optimized and tightly

controlled. The multiplex RT-PCR developed in this study amplified *sst1*, 2 and 5 and HPRT (a house-keeping gene (Foss *et al.* 1998)) transcripts from whole pituitary extracts in the same amplification reaction. To determine the cycle numbers that would correspond to the parallel amplification range of all PCR products within each reaction, PCR was performed between 21 and 36 cycles. All PCR products were amplified with similar efficiency between 24 and 30 cycles, as shown by the linear portions of the corresponding curves depicted in Fig. 1. Inasmuch as similar results were obtained in at least three independent experiments it can be assumed that: (i) there is no competition between primer sets; (ii) reagents are not limiting; and (iii) amplification efficiencies are similar for all transcripts within each reaction. To verify that the *sst*/HPRT ratio provides an accurate assessment of the level of *sst1*, *sst2* and *sst5* mRNA, varying volumes of the RT reaction representing 0.025–0.15 µg total RNA were added to the PCR reaction. Results showed that the ratios between *sst1*/HPRT, *sst2*/HPRT and *sst5*/HPRT remained constant in this concentration range (data not shown). It should also be noted that the expression level of HPRT represents an appropriate endogenous control since HPRT did not significantly vary between the various treatment groups in this study (data not shown). Accordingly, all subsequent PCR amplifications were performed with 0.1 µg total RNA equivalent (2 µl of the RT reaction) and at 27 cycles, which represents the midpoint of the linear portion of the curve.

Figure 1 Amplification kinetics of cDNA for pig pituitary SRIF receptor subtypes (*sst1*, *sst2* and *sst5*) and an endogenous internal standard (HPRT) using multiplex RT-PCR. One microgram of total pituitary RNA was reverse transcribed using random hexamer priming. cDNA was amplified by PCR in a single sample, in the presence of radiolabeled [α -³²P]deoxy-CTP, using specific primers for *sst1*, *sst2*, *sst5* and HPRT. Refer to Table 1 for specific primer sequences and product sizes. The radiolabeled PCR products were separated on a 5% polyacrylamide gel. The gel was dried on chromatography paper and exposed to a phosphorimage screen. The signal intensities (pixel densities) of the PCR products (upper panel) were measured by image analysis software. All PCR products were of the expected size. Amplifications of *sst1*, *sst2*, *sst5* and HPRT were parallel between 24 and 30 cycles (lower panel). Similar results were obtained in two additional, separate experiments. Accordingly, all subsequent PCR amplifications were performed at 27 cycles.

Effects of SRIF on SRIF receptor subtype mRNA levels in pig pituitary cell cultures

Having established a valid RT-PCR methodology to quantify pig *sst* mRNA levels, we first sought to ascertain the acute homologous regulation of *sst* gene expression. To this end, porcine pituitary cells were treated with two doses of SRIF (high, 10^{-7} M; and low, 10^{-13} M), which have been

previously shown to evoke different GH secretory responses in porcine somatotropes (Ramírez *et al.* 1997, 1998, 2002). Specifically, these were a high SRIF dose that inhibits GHRH-induced GH release without affecting basal GH secretion, and a low SRIF dose that increases GH release in the absence of GHRH. Results from these experiments are presented in Fig. 2, showing a representative gel picture (top panel) and the averaged results of three independent experiments (bottom graph). As can be observed in Fig. 2, a 4 h treatment with 10^{-7} M SRIF increased mRNA levels of *sst1*, *sst2* and *sst5* to 185, 164 and 455% of vehicle-treated controls respectively. The low dose of SRIF also enhanced *sst1* mRNA levels (239%) to values comparable with high-dose SRIF, but had no significant effect on *sst2* and *sst5* mRNA levels.

Effects of forskolin on SRIF receptor subtype mRNA levels in cultured pig pituitary cells

Since our laboratory has previously demonstrated that SRIF (at high and low doses) can stimulate cAMP production in a subpopulation of porcine pituitary somatotropes (Ramírez *et al.* 2002), it was of interest to determine if forskolin, a synthetic compound that increases cAMP levels by directly stimulating adenylyl cyclase activity, could mimic the effects of SRIF on SRIF receptor subtype expression. As shown in Fig. 2 (top, representative gel picture) and Fig. 3 (averaged results), treatment with forskolin (10 μ M; 4 h) increased *sst1* and *sst2* mRNA levels, without altering *sst5* expression, suggesting that some but not all of the actions of SRIF on the expression of its own receptor subtypes may be mediated via activation of the

Figure 2 Effects of acute, *in vitro* SRIF treatment on mRNA levels of pig pituitary SRIF receptor subtypes *sst1*, *sst2* and *sst5*. Dispersed pig pituitary cells were cultured for 4 h with two distinct SRIF doses (high, 10^{-7} M; or low, 10^{-13} M), and SRIF receptor subtype mRNA levels were determined by multiplex RT-PCR. The upper panel shows the image obtained in two representative experiments. Receptor-specific band intensities were determined and adjusted by the signal intensity for HPRT. The averaged results were then calculated and expressed as a percentage of vehicle-treated control levels (bottom panel; means $\pm$ S.E.M.; $n=3$ separate experiments, each containing three wells/group per experiment). * $P<0.01$ vs corresponding control.

Figure 3 Effects of acute, *in vitro* treatment with the cAMP-elevating agent forskolin on mRNA levels of pig pituitary SRIF receptor subtypes *sst1*, *sst2* and *sst5*. Dispersed pig pituitary cells were cultured for 4 h with forskolin (10 μ M), and receptor mRNA levels were determined by multiplex RT-PCR. Receptor-specific band intensities were adjusted by the signal intensity for HPRT. Results are expressed as a percentage of vehicle-treated control levels and represent the means $\pm$ S.E.M. ($n=3$ separate experiments, each containing three wells/group per experiment). * $P<0.01$ vs corresponding control.

Figure 4 Expression of pig pituitary SRIF receptor subtypes *sst1*, *sst2* and *sst5* in response to acute GHRH or ghrelin treatment. After 3 days of culture, dispersed pig pituitary cells were incubated with GHRH (10⁻⁸ M) or ghrelin (10⁻⁶ M) for 4 h, and SRIF receptor subtype mRNA levels were assessed by multiplex RT-PCR. Receptor-specific band intensities were adjusted by the signal intensity for HPRT. Results are expressed as a percentage of vehicle-treated control levels and represent the means $\pm$ S.E.M. ($n=3$ separate experiments, each containing three wells/group per experiment). * $P<0.01$ vs corresponding control.

cAMP/protein kinase A (PKA) intracellular signaling pathway.

Effects of GHRH and ghrelin on SRIF receptor subtype mRNA levels in cultured pig pituitary cells

As in other species, GHRH has been shown to stimulate GH release by increasing cAMP production in pig somatotropes (Ramírez *et al.* 1999). Additionally, we recently observed that ghrelin also augments cAMP and requires the activation of adenyl cyclase and PKA to stimulate GH secretion from cultured pig pituitary cell cultures (Malagón *et al.* 2003). Hence, we reasoned that if cAMP were the exclusive regulator of SRIF-mediated induction of *sst1* and *sst2* mRNA levels, GHRH and ghrelin would have comparable effects. Acute treatment (4 h) of pig pituitary cell cultures with GHRH (10⁻⁸ M) and ghrelin (10⁻⁶ M) showed this was not the case. As

depicted in Fig. 4, GHRH and ghrelin reduced *sst5* mRNA levels, but did not significantly modify the expression of *sst1* and *sst2*.

Discussion

This report demonstrates that SRIF can upregulate *sst1*, *sst2* and *sst5* mRNA levels when applied to porcine pituitary cell cultures in nanomolar concentrations. The stimulatory actions of SRIF on its own receptor synthesis have also been reported using a rat pituitary cell line, GH₃, where acute (2 h) and long-term (>24 h) treatment with SRIF (10⁻⁶ M) elevated *sst1*, *sst3*, *sst4* and *sst5* mRNA levels (Bruno *et al.* 1994a, Berelowitz *et al.* 1995). However, *sst2* mRNA levels in the aforementioned study were only observed to increase following acute SRIF exposure, but decreased with prolonged exposure. Short-term SRIF (4 h, 1 and 100 nM) treatment also increased SRIF receptor mRNA levels in primary rat pituitary cell cultures

but this increase was confined to the sst3 and sst5 receptor subtypes (Park *et al.* 2002). *In vivo*, i.v. treatment with octreotide, an sst2/5-preferring SRIF receptor agonist, enhanced sst1 and sst2 mRNA levels in the arcuate nucleus of the rat, while sst1 and sst2 mRNA levels of the pituitary were insensitive to treatment (Tannenbaum *et al.* 2001). Taken together, these results suggest that SRIF plays an overall stimulatory role on its own receptor synthesis; however, the regulation of specific receptor subtypes is dependent on the species, the tissue type, and the source of cells used (primary vs clonal cell lines).

Interestingly, a concentration of SRIF as low as 10^{-13} M, which stimulates GH secretion from cultured pig somatotropes (Ramírez *et al.* 1998), increased sst1 expression, but did not alter sst2 or sst5 mRNA levels. One can argue that the lack of an effect of low-dose SRIF on sst2 and sst5 mRNA levels may be explained by a lower sensitivity of these particular receptor subtypes to stimulation by their own ligand. An alternative explanation is that SRIF can activate distinct signaling mechanisms depending on the dose used, where the differential activation of these intracellular signaling pathways would have variable effects on SRIF receptor subtype expression. In support of the latter hypothesis, our laboratory has reported that SRIF differentially regulates the cAMP/PKA intracellular signaling in pig pituitary cell cultures depending on the dose used and the specific subpopulation of pituitary cells employed. At doses in the nanomolar range, SRIF inhibits GHRH-stimulated cAMP-initiated GH release in heterogeneous pig pituitary cell cultures (Ramírez *et al.* 2002), as observed in pituitary cell cultures from other mammalian species (Reisine & Bell 1995, Giustina & Veldhuis 1998, Meyerhof 1998, Patel 1999, Csaba & Dournaud 2001). However, at doses in the picomolar range, SRIF stimulates GH release from pig pituitary cells (Ramírez *et al.* 1998). The stimulatory action of SRIF is attributed to a subpopulation of porcine somatotropes that accounts for over 40% of the total somatotrope population (Castaño *et al.* 1996, Ramírez *et al.* 1997, 2002). This subpopulation responds to both high- and low-dose SRIF treatment by an increase in cAMP accumulation. In fact, the stimulatory effect of SRIF on GH release from these cells can be blocked by inhibitors of adenylyl cyclase activity (Ramírez *et al.* 2002).

In the present study we sought to determine if increasing intracellular cAMP by forskolin activation of adenylate cyclase could mimic the stimulatory actions of SRIF on its own receptor expression in porcine pituitary cultures. As with high-dose SRIF, forskolin augmented mRNA levels of sst1 and sst2, but did not alter sst5 expression, suggesting sst1 and sst2 gene expression or mRNA stability is enhanced by a cAMP-dependent pathway, whereas SRIF stimulation of sst5 expression is mediated by a cAMP-independent mechanism. In support of the cAMP-dependent regulation of pituitary sst1 and sst2 expression, similar results have been reported in primary rat pituitary cell cultures; however, in this species, forskolin suppressed sst5 mRNA levels, as well as those for sst3 and sst4 (Park *et al.* 2000). Forskolin has also been shown to increase the expression of sst2 in AtT-20 cells, a mouse corticotrope cell line (Patel *et al.* 1993). However, forskolin failed to alter the activity of a human sst2 promoter-driven reporter plasmid transfected in the rat pituitary cell line, GH₄C₁ (Petersenn *et al.* 1999). In the latter study, it is difficult to determine if the absence of a forskolin effect is due to species-dependent regulation of this SRIF receptor subtype or due to the lack of the appropriate regulatory elements in the heterologous promoter/reporter system. To date, CREs have been shown to be located in promoter regions of the human (Petersenn *et al.* 1999) and mouse (Woltje *et al.* 1998, Kraus *et al.* 2000) sst2 genes and rat sst3 gene (Glos *et al.* 1998, Baumeister & Meyerhof 2000), suggesting cAMP can regulate gene transcription of at least some SRIF receptor subtypes, by activation of cAMP-mediated PKA phosphorylation and binding of the CREB transcription factor (Mayr & Montminy 2001).

The inability of forskolin to mimic the stimulatory actions of SRIF on sst5 mRNA levels suggests that SRIF enhanced the expression of this SRIF receptor subtype via a cAMP-independent pathway. It is interesting to note that the cAMP-dependent regulation of sst5 appears to be the most varied among experimental models. In the rat, forskolin was shown to inhibit sst5 mRNA levels (Park *et al.* 2000), while in a transient transfection system dibutyryl cAMP increased the activity of a human sst5 promoter/reporter construct in GH3 cells, a rat pituitary cell line (Greenwood *et al.* 1994). Of the sst5 promoter regions examined (human (Greenwood *et al.* 1994,

Petersenn *et al.* 2002a), mouse (Gordon *et al.* 1999)) none has been shown to contain CREs, providing further evidence in favor of a cAMP-independent (or, at least, CRE-independent) regulatory mechanism possibly involving a yet to be identified intracellular signaling pathway. Clues to this alternative mechanism may be provided by the evidence generated in the pig, where we have observed that SRIF activates phospholipase C (PLC) signaling in a subpopulation of porcine pituitary somatotropes, although this activation does not seem to influence SRIF-induced GH release (Ramírez *et al.* 2002). Taking into account the well-established link between PLC and protein kinase C (PKC) activation, a direct regulation of *sst5* gene expression by PLC/PKC might be envisioned given the fact that the mouse *sst5* promoter (Gordon *et al.* 1999) contains multiple AP-1 (AP, activator protein) sites while the human *sst5* promoter contains both AP-1 and AP-2 sites (Greenwood *et al.* 1994). AP-1 and AP-2 sites have also been identified on the human *sst4* (Petersenn *et al.* 2002b), and rat *sst1* and *sst3* promoters (Hauser *et al.* 1994, Glos *et al.* 1998). These response elements have been shown to be the site of c-Fos/Jun heterodimer binding and transcriptional regulation of other genes activated by phorbol ester stimulation of PKC (Karin *et al.* 1997). In line with this concept, activation of PKC via phosphoinositide 3-kinase by SRIF in the A431 cervical carcinoma cell line results in increased signaling through the kinases Erk/MAPK (Stetak *et al.* 2001), which are functionally linked to transcriptional activation via AP-1 sites (Karin *et al.* 1997). Nevertheless, it should also be noted that cAMP can activate this same pathway (Gutkind 2000), an example of cross-talk between ligand-activated G-protein intracellular signaling pathways.

In an attempt to better understand the relative contribution of ligand/receptor activation of cAMP-dependent and -independent pathways to the regulation of pig SRIF receptor subtype expression, we examined the effects of GHRH and ghrelin in this regard. In the pig, as in other mammals studied to date, GHRH acutely stimulates cAMP production, which is required for maximal GH release (Giustina & Veldhuis 1998, Ramírez *et al.* 1999). In addition, ghrelin also raises cAMP and activates both cAMP/PKA and PLC/PKC intracellular signaling in pig pituitary cell cultures and both pathways appear to be

required for maximal GH release (Malagón *et al.* 2003). Despite the ability of both GHRH and ghrelin to activate a cAMP-dependent cascade in the pig pituitary, these two GH regulatory peptides did not mimic the actions of SRIF and forskolin on SRIF receptor subtype mRNA levels in the present study. In fact, both GHRH and ghrelin suppressed *sst5* mRNA levels without significantly altering *sst1* and *sst2* expression. The stark contrast between the actions of SRIF and forskolin vs GHRH and ghrelin on SRIF receptor mRNA levels brings into question the simplified view of one intracellular signaling pathway equals one action. The divergent effects of peptides that stimulate the same intracellular signaling systems within the same cell may be explained by intracellular compartmentalization of specific ligand/receptor signaling events, where particular receptor subtypes are isolated to microdomains of the cell, and their activation of the same intracellular signal can lead to opposite actions depending on its relative 'position' within the cell (Sim & Scott 1999, Schwartz 2001, Cordeaux & Hill 2002). Another possibility to explain the divergent effects of SRIF, GHRH and ghrelin on SRIF receptor expression may be that they are due to activation of yet to be identified intracellular signaling pathways which may act alone or in combination with known signaling events to mediate SRIF receptor expression.

As with the species-dependent effects of SRIF on its own receptor expression, our data generated using pig pituitaries make it clear that heterologous regulation of *sst1*, *sst2* and *sst5* expression are also species-dependent. In contrast to the exclusive inhibitory actions of GHRH and ghrelin on pig *sst5* expression, in the rat GHRH increases *sst1* and *sst2* mRNA levels and suppresses *sst5* mRNA levels *in vivo* and *in vitro*, while L-692, 585, a synthetic GHS-R ligand, had no effect (Park *et al.* 2000). The effects of GHRH on SRIF receptor subtype expression in the rat are thought to be mediated by cAMP, since forskolin mimicked the effects of GHRH *in vitro* (Park *et al.* 2000). However, in the present study using pig pituitary cell cultures, a direct correlation between GHRH and forskolin actions on SRIF receptor expression was not observed, thereby bringing into question the simple interpretation of the rat data.

The ultimate question is: what is the physiological impact of homologous and heterologous regulation of pituitary SRIF receptor level? With

respect to homologous regulation of SRIF actions, Presky & Schonbrunn (1988) and Berelowitz *et al.* (1995) reported that pretreatment of the rat pituitary cell lines for 24 h doubled SRIF receptor numbers as assessed by radiolabeled ligand binding without altering receptor affinity. The increase in ligand binding observed in these studies is likely due to stabilization of the SRIF receptor protein on the cell surface and not due to new receptor synthesis, in that cycloheximide, a protein synthesis inhibitor, did not disrupt the stimulatory effect of SRIF on subsequent ligand binding (Presky & Schonbrunn 1988). Although new receptor synthesis does not appear to play a role in SRIF-mediated upregulation of its own receptors in pituitary cell lines, we cannot exclude that this mechanism operates in primary pituitary cell cultures. Nonetheless, an upregulation of receptor synthesis or receptor protein stability may be critical in counteracting the well-characterized rapid internalization and/or desensitization by phosphorylation of SRIF receptors following ligand binding (Reisine & Bell 1995, Meyerhof 1998, Patel 1999, Csaba & Dournaud 2001). Conversely, downregulation of SRIF receptor expression by the GH-stimulatory peptides, GHRH and ghrelin, may act to ultimately augment their actions, because SRIF, at high doses, is a well-characterized inhibitor of both GHRH (Ramírez *et al.* 1998) and ghrelin (Malagón *et al.* 2003) function in pigs, as in other species (Reisine & Bell 1995, Giustina & Veldhuis 1998, Meyerhof 1998, Patel 1999, Csaba & Dournaud 2001, Yamazaki *et al.* 2002), most likely through its well-known opposing actions on adenylyl cyclase activity and calcium channel activation (reviewed in Giustina & Veldhuis 1998, Meyerhof 1998, Patel 1999).

In conclusion, this study demonstrates that pig pituitary sst1, sst2 and sst5 respond differentially to the acute actions of SRIF, GHRH and ghrelin and, unlike in other species studied to date, some of these responses do not appear to be mediated via cAMP. Accordingly, it seems reasonable to propose that the expression of SRIF receptors in pig pituitary cells is under a complex, species-specific and receptor subtype-selective regulation by GH-regulatory peptides that activate G-protein coupled receptor intracellular signaling cascades. Furthermore, given the distinct pattern of response of the three SRIF receptor subtypes to SRIF, GHRH and ghrelin, it seems highly unlikely that sst1, sst2 and

sst5 convey the same signals or exert identical biological effects in response to SRIF in porcine somatotropes. The multiplex RT-PCR assay developed herein to measure changes in SRIF receptor subtype expression, coupled with studies underway in our laboratory to clone and characterize all of the genes encoding pig SRIF receptor subtypes, paves the way to investigate new aspects of their expression (long-term changes, metabolic control and role of sex steroids), and will thus provide novel information to help understand their distinct regulation, and the precise signaling mechanisms underlying such control.

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