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Peptides of the Growth Hormone-Releasing Hormone Family

Differential Expression in Rat Testis

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Growth hormone-releasing hormone (GHRH) belongs to a family of peptides expressed at high levels in the brain and digestive system of mammals. We have identified GHRH mRNA and peptide in rat and human germ cells, and detected a GHRH receptor mRNA in Sertoli cells. GHRH treatment of cultured Sertoli cells results in accumulation of cAMP and increased expression of *c-fos* and stem cell factor (SCF), two factors critical for normal germ cell development. The current study was designed to localize within testis the transcription of other members of the GHRH family, and their receptors, and to determine if they also stimulate SCF. RNAs from separated testicular cells were amplified by comparative reverse transcription-polymerase chain reaction (RT-PCR). Southern blot analysis of the PCR products verified the presence of five GHRH family peptide and receptor transcripts in distinct testicular cell types. Transcripts encoding VIP and glucagon, and the receptors for pituitary adenylate cyclase activating peptide (PACAP) and glucagon, were detected predominantly in Leydig cells. In contrast, expression of GHRH, PACAP, secretin, and secretin receptor predominated in germ cells. Receptors for GHRH and VIP were expressed equally in all testicular cell types. To determine if, like GHRH, any of these other peptides activate Sertoli cell expression of SCF, primary Sertoli cell cultures were treated for 4–6 h with 10 or 100 n*M* of each individual factor. There was no consistent stimulation of SCF mRNA by VIP, PACAP, glucagon, or secretin. Differential expression of these peptides and their receptors suggests that they may each have unique paracrine functions within the testis.

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Introduction

Growth hormone-releasing hormone (GHRH) belongs to a family of homologous peptides that includes vasoactive intestinal peptide (VIP) and its related peptides histidine-isoleucine (PHI) and histidine-methionine (PHM); pituitary adenylate cyclase activating peptide (PACAP) and its related peptide (PRP); glucagon and the glucagon-like peptides (GLP-1 and GLP-2); gastric inhibitory peptide (GIP); and secretin. Several members of this peptide family have been detected in testis (Hakanson et al., 1982; Hueso et al., 1987, 1989; Berry and Pescovitz, 1988; Arimura et al., 1991; Shivers et al., 1991; Berry et al., 1992; Heindel et al., 1992; Ohkubo et al., 1992; Ohta et al., 1992; Spengler et al., 1993; Srivastava et al., 1993a, 1994; Shioda et al., 1994; Monts et al., 1995), and have been shown to affect testicular cell function (Kasson et al., 1986; Hueso et al., 1987; Heindel et al., 1992).

We recently localized mRNAs for secretin and a secretin receptor to rat spermatogenic cells (Monts et al., 1995). GHRH has been localized to spermatogenic cells (Pescovitz et al., 1990; Srivastava et al., 1993b) and Leydig cells (Ciampani et al., 1992), and has stimulatory effects on Leydig cell function (Ciampani et al., 1992). We have detected a GHRH receptor mRNA in Sertoli cells (Srivastava et al., 1994), and found that in vitro GHRH stimulates Sertoli cell expression of mRNAs for *c-fos* and stem cell factor (SCF) (Srivastava et al., 1993a), which are among the few intratesticular factors known to be essential for normal spermatogenesis (Geissler et al., 1988; Huang et al., 1990; Johnson et al., 1992).

The precise intratesticular localization of the entire GHRH family of peptides is largely unknown, and no single study has compared the differential expression of each ligand and its receptor. We designed this study to locate the intratesticular site of transcription of the GHRH family of peptides and receptors, and to determine if they share functional Sertoli cell activity with GHRH.

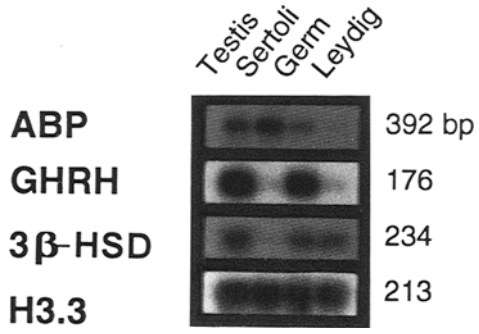

Fig. 1. Cell-specific products in separated testicular cells by RT-PCR analysis. Total RNAs from whole testis, Sertoli, germ, and Leydig cells were amplified by comparative RT-PCR as described under Methods. The PCR products were separated by electrophoresis through 1.8% agarose gels, transferred to nylon membranes, and hybridized to ^{32}P-labeled oligonucleotide probes from the sequences amplified. Histone 3.3 (H3.3) was used as a control for the efficiency of the RT-PCR reaction.

Results

To verify the purity of the separated cell populations, cell-specific transcripts were amplified by comparative RT-PCR. Intron-spanning primers were designed to amplify mRNAs for androgen binding protein (ABP), GHRH, and 3β-hydroxysteroid dehydrogenase (3β-HSD). These were used to detect potential contamination by Sertoli, germ, and Leydig cell fractions, respectively. Southern blot analysis indicated the presence of detectable Sertoli and Leydig cells in the germ cell fraction (Fig. 1), but Leydig and Sertoli cell fractions were nearly pure.

Members of the GHRH family of peptides and their receptor mRNAs were localized within testis by comparative RT-PCR analysis of RNA from whole testis, Sertoli, germ, and Leydig cells. Histone 3.3 (H3.3) was used as a control for the efficiency of the RT-PCR reactions, and to indicate equal gel lane loading and transfer for Southern blots.

All reactions amplified products of only the expected sizes, as determined by Southern blot analysis. This indicates absence of genomic contamination of the RNAs, since the PCR primers were designed such that the amplified sequences spanned introns.

Transcripts encoding VIP and glucagon and the receptors for PACAP and glucagon were detected predominantly in Leydig cells (Fig. 2). In contrast, expression of GHRH, PACAP, secretin, and secretin receptor predominated in germ cells. Receptors for GHRH and VIP were expressed essentially equally in all testicular cell types. H3.3 hybridization signal was equally intense in all lanes.

We had previously shown that GHRH activates expression of SCF in primary Sertoli cell cultures. In preliminary studies to determine specificity of function for GHRH family peptides in testis, primary Sertoli cell cultures were treated for 4–6 h with 10 or 100 n*M* of each GHRH family peptide, or with control medium. By Northern blot analysis, VIP, PACAP, glucagon, and secretin did not activate SCF expression. In contrast, only GHRH consistently stimulated SCF mRNA accumulation, by approx fivefold over control (Fig. 3). Expression of γ-actin was not affected by any of the factors (negative control).

Discussion

This is the first comprehensive study to compare the intratesticular localization of transcripts for the GHRH family of peptides and their receptors. Given the critical importance of germ cell development, we have hypothesized that members of the GHRH family of peptides, along with the many other neuropeptides, growth factors, transcription factors, and cytokines known to exist in testis, interact in a complex intratesticular network with sufficient functional redundancy to ensure normal progression of spermatogenesis (Pescovitz et al., 1994). There exists a large body of evidence that GHRH gene family peptides have similar structural conformations and tissue distributions, exhibit crossreactivities, and are of common ancestral origin, as reviewed (Campbell and Scanes, 1992).

Localization of GHRH mRNA to germ cells by RT-PCR is consistent with previous findings obtained by Northern blot analysis and *in situ* hybridization (ISH) (Srivastava et al., 1993b). We had previously found GHRH receptor message predominating in Sertoli cells (Srivastava et al., 1994), but detected it in all cell types in the current study. This result is supported by previous reports that demonstrated GHRH binding to Leydig cells (Ciampani et al., 1992).

VIP mRNA was most abundant in Leydig cells. VIP receptor mRNA was amplified from all cell types. VIP binding sites have previously been detected on rat Leydig cells (Hueso et al., 1989; Ciampani et al., 1992), where VIP stimulation activates cAMP accumulation (Hueso et al., 1987; Ciampani et al., 1992) and androgen biosynthesis (Kasson et al., 1986).

RT-PCR localization of PACAP mRNA to germ cells is in agreement with prior ISH findings (Shioda et al., 1994). We detected, predominantly in Leydig cells, three different-sized transcripts that correspond to the sizes of 3–5 known splice variants of the PACAP type I receptor (Spengler et al., 1993). We also amplified PACAP receptor transcripts from germ cells, where binding of PACAP has previously been reported (Shivers et al., 1991). In our study, Sertoli cells also contained detectable PACAP receptor mRNA. This is consistent with studies in which PACAP has been found to stimulate rat Sertoli cell functions, including cAMP accumulation (Heindel et al., 1992).

Glucagon mRNA was detected exclusively in Leydig cells, which also contain the most abundant transcripts for glucagon receptor. To our knowledge, this is the first study investigating the presence of glucagon in testis.

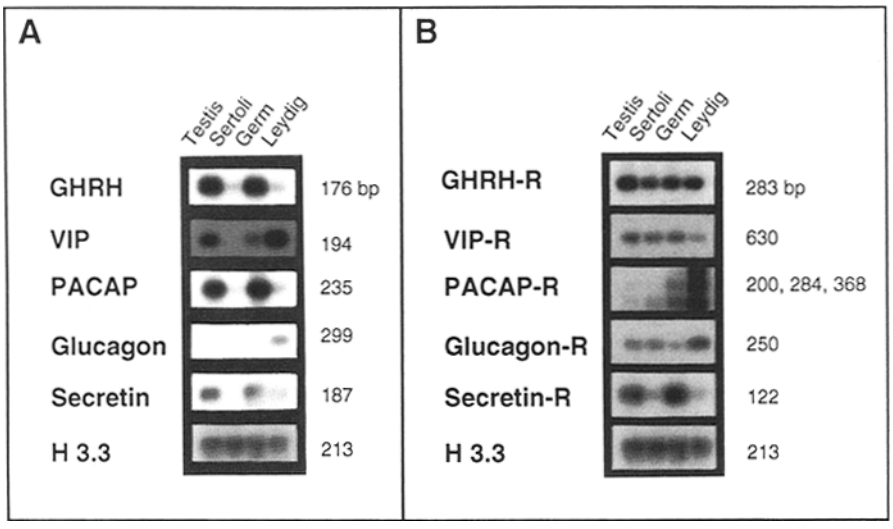

Fig. 2. GHRH family of peptides in separated testicular cells by RT-PCR analysis. Total RNAs from whole testis, Sertoli, germ, and Leydig cells were amplified, separated, transferred, and probed as described under Methods. **(A)** Amplified peptide messages and **(B)** Amplified receptor messages, using the primers described under Methods. This figure is representative of at least three separate experiments, using tissues from at least three different rats.

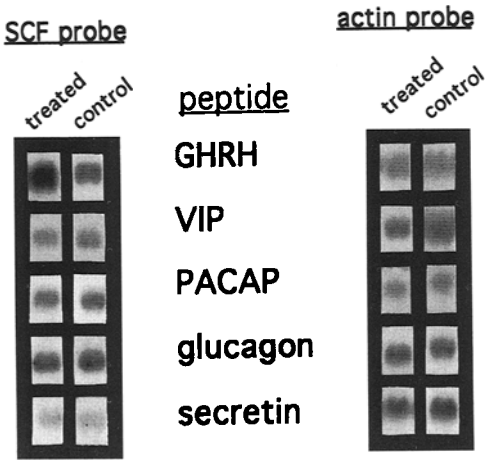

Fig. 3. Stimulation of Sertoli cell gene expression by GHRH family peptides, by Northern blot analysis. Sertoli cells, isolated and cultured for 4 d, were treated with 10–100 n*M* hormone or control medium for 4–6 h. RNA isolation and Northern blot analysis (10 μg total RNA per lane) were performed as described under Methods. Ribosomal RNAs were used as size markers. This figure is representative of at least five separate experiments, using tissues from at least five different rats.

The predominant germ cell localization of transcripts for secretin and secretin receptor is in agreement with our previous findings obtained by RT-PCR and ISH (Monts et al., 1995). Secretin, VIP, glucagon, and PHM-27 have been shown to stimulate progesterone and testosterone production in neonatal rat testicular cells, with relative potency proportional to their homology to VIP (Kasson et al., 1986). Similarly, we had hypothesized intratesticular colocalization of mRNAs for GHRH family peptides that share the greatest sequence homology, but this was not found to be the case.

Previously, we had monitored the purity of testis cell fractions by phase contrast microscopy. In the current study, we attempted to refine this characterization by evaluating each cell fraction for specific gene expression by RT-PCR. We used 3β-hydroxysteroid dehydrogenase (3β-HSD) to verify the presence of Leydig cells; androgen binding protein (ABP) to verify the presence of Sertoli cells; and GHRH to verify the presence of germ cells.

These studies indicated that only the germ cell fraction contained detectable evidence of other cell types. Since spermatogenic cells do not thrive in primary culture, their RNA was harvested immediately following isolation of the cells. Therefore, presence of Sertoli and Leydig cells was minimized but not entirely removed. Sertoli and Leydig cell preparations contain no detectable contamination. These fractions are cultured 4 d and overnight, respectively, during which time contaminating cells do not remain viable and are removed when the culture medium is changed.

Unlike GHRH, the other peptides we tested did not significantly stimulate expression of SCF in Sertoli cells under similar conditions. This warrants further investigation, however, as we did discover detectable, but statistically insignificant, stimulation in some experiments. Thus, GHRH appears to be unique in its ability to regulate SCF. Also, this effect of GHRH is specific for SCF, as other transcripts (ABP, transferrin, sulfated glycoprotein-2, α-inhibin) remained unaffected by GHRH treatment (unpublished results).

In the adult rat, Sertoli cells comprise only 16.6–18.9% of the total seminiferous epithelium volume (Russell et al., 1990), so Sertoli cells are isolated from prepubertal rats in order to obtain sufficient yield and freedom from germ cell contamination. Thus the possibility remains that GHRH

Table 1
Intron-Spanning Primers for Comparative RT-PCR Analysis

mRNA	5' primer	3' primer
Androgen binding protein	5'-AAGCTGGTGGATGGAGCAGGAC-3'	5'-GTCCAGATGTCCTGGCTTCTGC-3'
GHRH	5'-CGGCATGCAGACGCCATCTTCA-3'	5'-ATGCTCTCCAGGGCCATCTGCT-3'
GHRH receptor	5'-CGATCGAGAGTGAAGGTGCTC-3'	5'-GCCACATTGACTCAACTGCAG-3'
3β-hydroxysteroid dehydrogenase	5'-GCTCTACTGGCTTGCCTTCCTG-3'	5'-AGTGTCTCCCTGTGCTGCTCCA-3'
VIP	5'-TTTTCACCAGCGACTACAGTAGACT-3'	5'-GGAGTTCAAGTATTTCTTCACAGCC-3'
VIP receptor	5'-ACATGCAGCACGCAGGTATCCA-3'	5'-CTCCCAGGTCTGAGGATACACT-3'
PACAP	5'-GGATCCTTGATAAGGCCTACCGCAAA-3'	5'-TGTTTATACCTTTTCCCTAGGACAGC-3'
PACAP receptor	5'-ATGACAGCACAGCTCTGTGGT-3'	5'-GTAGTGGATTCCGAAGAGTGG-3'
Glucagon	5'-CATTCACAGGGCACATTCACCAGT-3'	5'-GAGAAGGATCCATCAGCATGTCTG-3'
Glucagon receptor	5'-CACCTGAATCTCCACTGGAC-3'	5'-CTGTGTGCATGGAACATGGC-3'
Secretin	5'-GAAAATATTCCAGAGAACAGCGTG-3'	5'-TTGTTTCAGTCCACTCTGAATGGTC-3'
Secretin receptor	5'-CGCTCTTTGGCATCCACTACAT-3'	5'-TGAGGAAGCAGTAAAGGACAGC-3'
Histone 3.3	5'-GCAAGAGTGCGCCCTCTACTG-3'	5'-GGCCTCACTTGCCTCCTGCAA-3'

family peptides stimulate SCF expression, or some other function, in adult Sertoli cells. Evidence exists that testicular GHRH is developmentally regulated (Berry and Pescovitz, 1990) and under tissue-specific transcriptional control (Srivastava et al., 1995); testing for these attributes in other GHRH family peptides will be illuminating.

More precise intratesticular localization of GHRH family peptides, by immunohistochemistry and ISH, will also provide clues as to their possible functions. The GHRH family peptides have conserved alpha-helical conformations, with microheterogeneity within the helices, which may account for some of their divergent biological activities in the whole animal (Campbell and Scanes, 1992). Whether this predicts unique actions of each GHRH family peptide within testis remains a compelling question.

Materials and Methods

Animals

Male Sprague-Dawley rats obtained from Harlan (Indianapolis, IN) were killed by decapitation, and testes were rapidly dissected and decapsulated. All animals were maintained in accordance with the guidelines set by the NIH Guide for the Care and Use of Laboratory Animals, as well as the Animal Use and Care Committee of Indiana University School of Medicine.

Cell Fractionation and Sertoli Cell Culture

Germinal cells were isolated using sequential collagenase and trypsin treatment of adult testis essentially as described (Romrell et al., 1976). Leydig cells were isolated by collagenase treatment of adult seminiferous tubules followed by ficoll gradient purification (Janszen et al., 1976; Rommerts et al., 1985) using modifications previously described (Garrett and Douglass, 1989). Cell purity was monitored by phase contrast microscopy and verified by RT-PCR amplification of cell-specific transcripts. Sertoli cell cultures were prepared from 20–22-d-old rats (Dorrington and Fritz, 1975) and cultured at 32°C in Ham's F-12 medium supplemented with 9 m*M* HEPES, pH 7.5, 215 μg/mL L-glutamine, 50 U/mL penicillin, 50 μg/mL streptomycin, 50 μg/mL gentamicin, and 0.625 μg/mL fungizone. Medium was changed the 3rd d after plating. Twenty-four hours later, hormone treatments were initiated (for Northern blot analysis), or RNA was isolated (for RT-PCR).

Primers and Probes

PCR primers and oligonucleotides used for Southern blot analysis were synthesized using an Applied BioSystems Oligonucleotide Synthesizer (Foster City, CA). For comparative RT-PCR analysis, intron-spanning primers were selected as shown in Table 1.

Most probes were 30-bp oligos from the amplified sequences, which were ^{32}P-end-labeled using a kit from Gibco BRL (Gaithersburg, MD). However, PCR products we had previously cloned and sequenced were used as probes for secretin and secretin receptor (Monts et al., 1995) and GHRH receptor (Srivastava et al., 1994). Histone 3.3 primers generate a 213-bp DNA fragment after PCR, as described previously (Kelley et al., 1993), from which a probe was made for hybridization to Southern blots. These probes, and the probes for Northern blot analysis, were ^{32}P-labeled by the hexanucleotide primer method (Feinberg and Vogelstein, 1983), using a kit from Stratagene (La Jolla, CA).

For Northern blot analysis, the SCF probe was a 0.6-kb *Hind* III-*Sac* II fragment from a plasmid containing a murine cDNA (kindly provided by David A. Williams, Indiana University Medical Center). A human γ-actin probe, which crosshybridizes with rat actin, was obtained from Winston A. Salser (UCLA).

Comparative Reverse Transcription (RT) and Polymerase Chain Reaction (PCR) Analysis

RNA was isolated by the guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987) for subsequent RT-PCR (Wilson et al., 1992; Kelley et al., 1993). Three micrograms of total RNA were used for reverse transcription (RT); 2.5 μL of the RT reaction from each sample were incubated in 10 m*M* Tris-HCl (pH 8.3), 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 200 μ*M* of each deoxynucleotide triphosphate, 0.01% gelatin, 0.01% Tween-20, 0.01% Nonidet P-40, 2 μ*M* of each primer, and 2 U Taq polymerase (US Biochemical, Cleveland, OH). The polymerase amplification was carried out in a PTC-100 Thermal Controller (Medical Research, Inc.), using 25 cycles of amplification (95°C, 30 s; 55°C, 1 min; 72°C, 2 min), followed by 10 min at 72°C. For negative controls, each RNA was treated as just described, but with reverse transcriptase omitted; also, RT-PCR reactions were performed with no RNA, using each primer pair.

To allow for comparison of mRNA levels between cell types, PCR reactions were terminated before amplification was complete. This was quantitated, in preliminary experiments, by running aliquots of the reaction mixture through agarose gels after 15, 20, 25, 30, and 35 cycles of amplification. For the final experiments, 25 cycles were selected, because at this point amplification was not yet complete. Thus, specific cell fractions yielded PCR products in amounts corresponding to the mRNA levels in those cells.

Southern Blot Analysis

After PCR, 20 μL of the reaction products were separated by electrophoresis using 1.8% agarose gels and transferred to MagnaGraph nylon membranes (Micron Separations, Inc., Westboro, MA). The blots were hybridized to either histone 3.3, ligand, or receptor ^{32}P-labeled oligonucleotide probes under conditions previously described (Srivastava et al.,1993b).

Northern Blot Analysis

After incubation with hormone for 4–6 h, Sertoli cells were harvested by plate scraping, and the RNA isolated by the guanidinium thiocyanate-phenol-chloroform extraction method. For Northern analysis, 10 μg of total RNA from each sample were separated by electrophoresis through a 1.2% agarose gel containing 6.7% formaldehyde in MOPS buffer and transferred to a nylon membrane. The blots were hybridized at 42°C overnight to SCF and γ-actin cDNA probes made by random primer method. The blots were washed with 0.1X SSC and exposed to film at –80°C. Autoradiograms were scanned on a Scanmaker II (Microtek, Torrance, CA), and data were quantitated using Sigma Scan (Jandel Scientific, San Rafael, CA), with normalization to actin.

Acknowledgments

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