Corticotropin-releasing hormone receptor expression and functional signaling in murine gonadotrope-like cells

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Abstract

Corticotropin-releasing hormone (CRH) is a key regulator of the mammalian stress response, mediating a wide variety of stress-associated behaviors including stress-induced inhibition of reproductive function. To investigate the potential direct action of CRH on pituitary gonadotrope function, we examined CRH receptor expression and second messenger signaling in aT3-1 cells, a murine gonadotrope-like cell line. Reverse transcriptase PCR (RT-PCR) studies demonstrated that aT3-1 cells express mRNA for the two CRH receptor subtypes, CRHR1 and CRHR2, with CRHR2 a as the predominant CRHR2 isoform. Stimulation of the cells with CRH or urocortin (UCN) resulted in rapid, transient increases in the intracellular levels of cAMP that were completely blocked by the addition of a-helical CRH 9-41 or astressin, non-selective CRH receptor antagonists. Stimulation of the cells with CRHR2-specific ligands, urocortin 2 (UCN2) or urocortin 3 (UCN3), resulted in rapid increases in intracellular cAMP levels to 50–60% of the levels observed with UCN. Treatment with a selective CRHR2 antagonist, antisauvagine, completely blocked UCN3-mediated increases in cAMP and significantly reduced, but did not completely block UCN-mediated increases in cAMP, demonstrating that both CRHR1 and CRHR2 are functionally active in these gonadotrope-like cells. Finally, UCN treatment significantly increased the transcriptional activity of the glycoprotein hormone a-subunit promoter as assessed by a-luciferase transfection assays. Together, these results demonstrate the functional signaling of CRH receptors in aT3-1 cells, suggesting that CRH may also modulate pituitary gonadotrope function in vivo.


Introduction

Corticotropin-releasing hormone (CRH) is a key regulator of the endocrine, behavioral, and autonomic components of the mammalian stress response. Within the endocrine hypothalamic–pituitary–adrenal axis, this 41-amino acid peptide is the key mediator of ACTH secretion. Hypothalamic CRH is released in response to stressful stimuli and carried to the anterior pituitary where it activates CRH receptors on anterior pituitary corticotropes, resulting in increased ACTH synthesis and secretion. ACTH acts on the adrenal cortex to increase glucocorticoid release; glucocorticoids then mediate many of the metabolic changes required to respond to the stressor. CRH is also expressed in many other sites in the central nervous system (CNS) where it is thought to act as a neurotransmitter to mediate changes in a wide variety of stress-associated behaviors. For example, CRH has been implicated in stress-induced alterations in anxiety-like behavior, feeding behavior, and locomotor activity (Dunn & Berridge 1990, Owens & Nemeroff 1991).

CRH has also been shown to play an important role in stress-induced inhibition of reproductive function. The i.c.v. injection of CRH suppresses the activity of the hypothalamic–pituitary–gonadal (HPG) axis in rats, resulting in decreased release of GNRH1 and LH (LHB; reviewed in Rivest & Rivier (1995)). Similarly, the repression of pulsatile LHB secretion by restraint stress, lipopolysaccharide, insulin-induced hypoglycemia, or fasting can be reversed by the icv injection of CRH receptor antagonists (Maeda et al. 1994, Li et al. 2005, 2006), suggesting that CRH receptor ligands mediate inhibitory effects of stress on the HPG axis via central pathways (reviewed in Rivest & Rivier (1995)). While studies suggest that systemic administration of CRH does not alter LHB secretion in rats (Rivier & Vale 1984), CRH treatment of primary rat pituitary cultures decreased basal LHB secretion (Blank et al. 1986). Numerous other hypothalamic peptides have also been shown to modulate gonadotropin levels by acting at the pituitary (reviewed in Evans (1999)). Thus, CRH and other members of the CRH family of peptides, urocortin (UCN), urocortin 2 (UCN2), and urocortin 3 (UCN3), may participate in the stress-mediated regulation of reproduction via multiple mechanisms and sites of action.
CRH and the urocortins mediate their effects via two distinct CRH receptors: CRHR1 and CRHR2 (reviewed in Dautzenberg & Hauger (2002), Grammatopoulos & Chrousos (2002), Bale & Vale (2004) and Hillhouse & Grammatopoulos (2006)). The receptors are ~70% identical at the amino acid level and are both members of the class B subfamily of seven transmembrane domain G-protein-coupled receptors. In most cell types, the activation of CRHR1 or CRHR2 by CRH or urocortins results in the activation of the Gs-adenyl cyclase pathway and increased cAMP levels; however, recent evidence has demonstrated that in some tissues and cell types both CRH receptors can couple to other G-proteins including Gq, Gi, and Go, resulting in the activation of various kinases including PKC, MAPK, and Akt1 (reviewed in Grammatopoulos & Chrousos (2002) and Hillhouse & Grammatopoulos (2006)). CRHR1 and CRHR2 differ in their pharmacological properties (reviewed in Dautzenberg & Hauger (2002), Grammatopoulos & Chrousos (2002), Bale & Vale (2004) and Hillhouse & Grammatopoulos (2006)). CRHR1 has very high affinity for both CRH and UCN (Vaughan et al. 1995). By contrast, CRHR2 has a significantly higher affinity (20- to 40-fold) for UCN than for CRH (Vaughan et al. 1995); UCN2 and UCN3 are relatively selective for CRHR2, and have been suggested to be CRHR2-specific ligands (Hsu & Hsueh 2001, Lewis et al. 2001, Reyes et al. 2001).

CRHR1 has been detected in numerous sites in the brain (i.e., cerebellum, cerebral cortex, amygdala, various limbic, and sensory nuclei), the intermediate lobe of the pituitary, and a subset of corticotropes in the anterior lobe of the rat pituitary (Potter et al. 1994, Van Pett et al. 2000). Recent studies from our laboratory have demonstrated Crhr1 mRNA not only in a subset of corticotropes but also in a subset of lactotropes and gonadotropes in murine anterior pituitary (Westphal et al. 2009). CRHR2 has been isolated in two alternatively spliced forms in the rodent (CRHR2α and CRHR2β which utilize alternative amino termini) and exhibits an mRNA expression profile that is distinct from that of CRHR1 (Chalmers et al. 1995, Lovenberg et al. 1995b). CRHR2α is expressed primarily in brain (Chalmers et al. 1995, Lovenberg et al. 1995a), while CRHR2β is found largely in the periphery, but is also detected in the choroid plexus and cerebral arterialies (Chalmers et al. 1995, Lovenberg et al. 1995a). Within the pituitary, CRHR2 is expressed predominantly in the posterior lobe; however, a very low but detectable level of Crhr2 mRNA is also detected in the anterior lobe by in situ hybridization (Van Pett et al. 2000, Kageyama et al. 2003). Consistent with the potential expression of CRHR2 in anterior pituitary, Kageyama and colleagues recently demonstrated CRHR2α mRNA in rat anterior pituitary and Crhr2 mRNA in rat gonadotropes (Kageyama et al. 2003). These results suggest that both CRHR1 and CRHR2 may bind CRH and urocortins not only in the CNS but also in the pituitary to modulate stress-related functions.

To investigate the potential action of CRH specifically on pituitary gonadotrope function, we examined the CRH receptor expression and functional signaling in αT3-1 cells, a murine gonadotrope precursor cell line. These cells were originally derived by targeted oncogenesis in transgenic mice (Windle et al. 1990) and they express GNRH1 receptors and the α-subunit of the glycoprotein hormone, but not LHB or FSHB. They exhibit a number of characteristics equivalent to gonadotropes in primary pituitary cultures, demonstrating the utility of these cells as a model system for the study of gonadotrope function (Horn et al. 1991).

Materials and Methods

Cell culture

αT3-1 cells were kindly provided by Dr Pamela Mellon (University of California, San Diego; Windle et al. 1990). They were maintained in DMEM (Life Technologies) plus 10% FCS (HyClone, Logan, UT, USA) at 37 °C in 5% CO2.

cAMP assays

αT3-1 cells were plated (300 000 cells/well) onto 6-well plates (Pacton 3046) and used 2–3 days after plating. The cells were washed two times with DMEM and preincubated for 45–60 min in DMEM containing 1 mM isobutylmethyl-xanthine (IBMX; Sigma) to inhibit cAMP phosphodiesterase activity. Preincubation media were removed and DMEM (1 ml) containing 1 mM IBMX and various concentrations of human/rat CRH, rat UCN, mouse UCN2, mouse UCN3, α-helical CRH (9-41), astressin (AST; American Peptide Company, Sunnyvale, CA, USA), or antisauvagine (Bachem, Torrance, CA, USA) were added. The cells were incubated at room temperature for the indicated lengths of time before the removal of stimulation media. Then the cells were immediately lysed and intracellular cAMP levels were determined. The cyclic AMP (³H) assay system (Amersham Biosciences) was used for the time-course, dose–response profiles with CRH and UCN, and α-helical CRH 9-41 and AST antagonist studies. In this assay, the cells were lysed and incubated for 16–24 h at −20 °C with 95% ethanol/20 mM HCl (1 ml/well). Cell lysates were then dried under vacuum, resuspended in 300 μl of 0.05 M Tris (pH 7.5), 4 mM EDTA, and cAMP levels were determined in 50 μl samples, according to the manufacturer’s protocol, using triplicate wells and duplicate assays for each time point or treatment value. Additional dose–response studies with UCN, UCN2, and UCN3 and antisauvagine antagonist studies were performed with the direct cAMP enzyme Immunoassay kit (catalog no. 901-066; Assay Designs Inc., Ann Arbor, MI, USA). After the removal of the stimulation media, the cells were lysed by 20-minute incubation in 1 ml of 0.1 M HCl. The cell lysates were centrifuged at 600 g at room temperature for 5 min and the supernatants were used directly in the assay (50 μl/well) or stored at −20 °C.

As above, duplicate assays and duplicate or triplicate wells were used for each treatment condition. Each experiment was performed at least two times; dose profiles were repeated three to five times.

RNA isolation and cDNA synthesis

Total RNA was isolated from mouse brain, hypothalamus, heart, and αT3-1 cells using TRIzol reagent (Invitrogen). Five micrograms of RNA were used for random hexamer-primed first-strand cDNA synthesis (Novagen Inc, Madison, WI, USA). To control for genomic DNA contamination, cDNA synthesis was performed in the presence and absence of SuperScript II Reverse Transcriptase (Life Technologies, Inc.) for each of the RNA samples. The products of cDNA synthesis were purified using QIAquick PCR Purification kit (Qiagen Inc).

RT-PCRs

RT-PCR was performed in a final volume of 25 or 50 μl using 1–3 μl cDNA product, 200 μM deoxynucleotides, 1–0 μM primers, Taq buffer with MgCl2, and 1–25 U Taq DNA polymerase or platinum Taq DNA polymerase (Invitrogen Inc). Cycle conditions are described below. Based on the mouse CRHR1 genomic structure (Ensembl ENSMUST00000093925), the primers for mouse CRHR1 were designed to span at least one intron in the genomic sequence. The primer pair for CRHR1 (Fig. 1A) was as follows: forward primer, 5′-GGA-TCA-GTA-GAG-GCT-CCT-3′ and reverse primer, 5′-GTT-CCA-GTG-GAT-GAT-GTT-CCT-3′ (amplifying a 394 bp fragment from nucleotides 109 to 502 of mouse Crhr1 cDNA, GenBank accession no. X72305). PCR amplification conditions were 34 cycles of 94 °C for 30 s, 69 °C for 30 s, and 72 °C for 30 s.

The genomic organization and the sequence of mouse CRHR2 was recently published (Chen et al., 2005). Exons 1 and 2 are spliced to exon 4 to produce CRHR2 β while exon 3 is spliced to exon 4 to produce CRHR2 α. Our primers for PCR were designed to span at least one intron. For CRHR2 α, the forward primer was 5′-TCG-CTG-GAG-GGC-ATT-3′ and the reverse primer was 5′-GTT-CCA-GTA-GAG-GAT-GTT-CCT-3′ (amplifying a 243 bp fragment from nucleotides 158 to 400 of mouse CRHR2 α cDNA, GenBank accession no. AY445512). For CRHR2 β, the forward primer was 5′-GCG-CTA-AGA-GAG-CCG-GAC-AGA-CCT-CCT-TTG-GA-3′ and the reverse primer was 5′-AGA-ATG-GTG-ATG-GTG-ATG-GTC-TGG-GTC-ATA-3′ (exons 1–7, amplifying 480 bp of the mouse CRHR2 β cDNA, GenBank accession no. X72305). PCR amplifications included 34 cycles of 92 °C for 60 s, 66 °C for 90 s, and 72 °C for 90 s.

All PCR products were electrophoresed and visualized on an ethidium bromide-stained 1-0% agarose gel. The 1 kb plus DNA ladder (Fig. 1A) or 1 kb DNA ladder (Fig. 1B and C; Invitrogen) was used for DNA size standards.

Transcript transfections and reporter constructs

Cultures of αT3-1 cells were plated onto 6-well plates (400 000 cells/well) in DMEM + 10% FCS + gentamicin (50 μg/ml). The cells were transfected 12–24 h later, using FuGENE (see the manufacturer’s recommendations; Roche) with 1·75 μg luciferase reporter DNA and 0·25 μg RSV-βgal DNA per well. The α-subunit–luciferase construct was kindly provided by Dr Sally Camper (University of Michigan) and contains 480 bp of the mouse glycoprotein hormone α-subunit promoter (−480 to +43 bp) fused to the luciferase reporter gene (Brinkmeier et al., 1998). RSV-βgal was used to normalize for transfection efficiency. For all studies, 10 μM forskolin (Calbiochem, San Diego, CA, USA) or various concentrations of UCN peptides were applied at 40 h post-transfection. The cells were harvested 4 h later (44 h post-transfection) in cold 1× PBS, pelleted, and lysed in 100 μl lysis buffer (0·25 M Tris (pH 8·0), 0·1 M EDTA, 15 mM MgSO4, 1 mM DTT, and 1% Triton X-100) and incubated on ice for 10 min. Lysates were centrifuged at 10 000 r.p.m. for 10 min at 4 °C and 5 μl of each supernatant were added to 100 μl of luciferase assay buffer (Cortright et al., 1997) and assayed for 30 s in a Turner 20/20 luminometer; β-galactosidase activity was assayed as described previously. Data are represented as fold induction over untreated vehicle control (untreated controls = 1·0). Experiments were performed in duplicate and each experiment was repeated at least three independent times. Fold inductions from three independent experiments were combined for the α-luciferase transfection data presented.

Data analysis and statistical methods

All data are presented as mean ± s.e.m. Statistical significance of the cAMP responses over time and in the presence of CRH receptor antagonists was determined by ANOVA with post hoc analysis using StatView (Abacus Concepts). Duplicate or triplicate wells were used for all cAMP assays and at least two to five independent assays were performed for each study. The EC50 values for CRH, UCN, UCN2, and UCN3 were determined by non-linear regression analyses using GraphPad Prism (version 3.0) software (GraphPad). Statistical analysis for the transient transfection studies was performed using a one–way ANOVA followed by multiple comparison post hoc analysis, where all selected groups were analyzed simultaneously. Analysis was performed using StatView and p values that reached 95% confidence levels are included in each figure legend.

Results

Expression of CRH receptor mRNA in αT3-1 cells

RT-PCR was utilized to characterize the expression of Crhr1 and Crhr2 mRNA in αT3-1 cells. cDNA synthesis reactions
were performed in the presence (+) and absence (−) of reverse transcriptase to provide controls for genomic DNA contamination of the cDNA. CRHR1 and CRHR2 PCR primer pairs were also specifically designed to span at least one intron. Mouse hypothalamus (Hy) and mouse total brain (Br) cDNA were used as positive controls for CRHR1 and CRHR2 respectively. As shown in Fig. 1A, the primer pair for CRHR1 demonstrates the presence of an appropriately sized PCR fragment in the cDNA from αT3−1 cells (αT+) and mouse hypothalamus (Hy+) (394 bp, Fig. 1A). No bands were seen in the minus reverse transcriptase lanes (−), suggesting the absence of genomic DNA contamination. RT-PCR with two additional primer pairs confirmed the presence of CRHR1 in αT3−1 cells (data not shown; Westphal et al. 2009).

RT-PCR was also performed using multiple sets of primer pairs for CRHR2. CRHR2 is expressed in two alternatively spliced forms in the rodent: CRHR2α and CRHR2β (14, 19). These alternatively spliced transcripts differ in the 5′-untranslated region and the NH2 terminus of the encoded protein. Primer pairs were selected that contained the 5′-forward primer within the alternatively spliced region of the transcript, providing specificity for the CRHR2α or CRHR2β mRNAs. The results shown in Fig. 1B demonstrate the presence of an appropriately sized fragment for CRHR2α (243 bp) in αT3−1 (αT+) and mouse total brain (Br+) cDNA. The 243 bp fragment from αT3−1 cDNA was subcloned; DNA sequence analysis confirmed that the fragment contained the CRHR2α cDNA sequence. Additional primer pairs also confirmed the presence of CRHR2α in αT3−1 cells (data not shown). The cDNA samples used for the PCR in Fig. 1B were also tested with several different CRHR2β primer pairs. While the 631 bp CRHR2β-specific PCR product is readily detected in total brain (Br+) (Fig. 1C) or mouse heart (not shown), CRHR2β is not detected in the same αT3−1 cDNA samples (αT+) that readily detected CRHR2α. Additional primer pairs also failed to demonstrate detectable levels of CRHR2β in the αT3−1 samples. Together, the data in Fig. 1A–C clearly demonstrate the presence of Crhr1 and CRHR2α mRNAs in αT3−1 cells, with non-detectable levels of CRHR2β transcripts.

Stimulation of cAMP production in αT3−1 cells by CRH and UCN

CRH receptors have been shown to couple to Gs in many tissues and cell lines, resulting in increased intracellular cAMP levels. To test the intracellular signaling of the CRH receptors in αT3−1 cells, the cells were treated with 200 nM CRH or UCN in the presence of 1 mM IBMX for various lengths of time and cAMP production was examined. UCN and CRH bind to both CRHR1 and CRHR2, although CRH binds to CRHR2 with a 14-fold lower affinity than CRHR1 (K i = 13 and 0.95 nM respectively), while UCN binds to both CRHR1 and CRHR2 with very high affinity (K i < 1·0 nM; Donaldson et al. 1996). As shown in Fig. 2, treatment of αT3−1 cells with CRH or UCN resulted in rapid increases in the intracellular cAMP levels. The CRH- and UCN-mediated increases in cAMP were maximal at 2–5 min and were significantly increased over the levels in vehicle-treated cells (dashed line). The increased cAMP levels were usually two- to fourfold over basal levels, and maximal levels of cAMP did not significantly differ between CRH and UCN treatments within the experiments. Interestingly, the intracellular cAMP levels decreased rapidly from peak values even in the presence of 1 mM IBMX.

Dose-dependent stimulation of cAMP production in αT3−1 cells by CRH and UCN

Dose–response studies were performed using various concentrations of CRH or UCN in the presence of 1 mM IBMX. As time-course experiments had shown maximal...
intracellular cAMP levels at 2–5 min after the addition of ligand, all treatments were for 3 min. As shown in Fig. 3, cAMP levels increased in a dose-dependent fashion in response to CRH or UCN treatment. CRH and UCN were similar in potency and showed equivalent maximum cAMP levels across multiple experiments. The half-maximal stimulatory (EC50) concentrations of CRH and UCN were not significantly different with the values of 14.5 nM and 5.1 nM respectively.

Inhibition of CRH- and UCN-mediated increases in cAMP with non-selective CRH receptor antagonists

We used CRH receptor antagonists (α-helical CRH 9-41 and astressin) that block both CRHR1 and CRHR2 (reviewed in Hauger et al. (2006)) to determine whether the CRH- and UCN-mediated increases in intracellular cAMP were specifically mediated by CRH receptors. The cells were pretreated for 1 h with 1 mM IBMX and fresh media were added that contained 100 nM CRH or UCN in the presence or absence of 10 μM α-helical CRH 9-41 (9-41, Fig. 4A) or 1 μM AST (Fig. 4B). Cells were also treated with vehicle or receptor antagonist alone. All treatments were for 3 min. α-Helical CRH 9-41 treatment alone showed a small but significant increase in the intracellular cAMP levels (Fig. 4A). CRH or UCN treatment alone also resulted in a significant increase in cAMP, while co-administration with α-helical CRH 9-41 reduced cAMP values to vehicle-treated levels. The α-helical CRH 9-41 results shown here are consistent with the previous studies that suggest that it is not a pure antagonist, demonstrating

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weak intrinsic agonist activity (Baldwin et al. 1991, Hauger et al. 2006). A second non-selective CRH receptor antagonist, AST (Fig. 4B), also showed complete suppression of the CRH- and UCN-mediated increases in cAMP, with no significant effect of AST alone. Together, these studies clearly demonstrate that CRH and UCN mediate significant increases in the intracellular cAMP levels that can be effectively inhibited by the CRH receptor antagonists that block both CRHR1 and CRHR2 signaling.

Contributions of CRHR2 to the cAMP signaling in αT3-1 cells

To determine the contribution of CRHR2 to the overall cAMP increases in αT3-1 cells in response to CRH ligands, we stimulated the cells with various doses of UCN2 and UCN3, CRHR2-specific ligands (Jahn et al. 2004). The EC50 values for cAMP stimulation for UCN2 and UCN3 were 20.3 ± 9.7 nM (n = 3) and 32.5 ± 8.2 nM (n = 3), not significantly different from each other or the EC50 value of CRH (21.2 ± 5.2 nM, n = 7). At maximal doses of UCN2 and UCN3 (100 nM), we observed increases in cAMP levels that reached 60.0 ± 7.6% (n = 6) and 54.3 ± 6.0% (n = 13) of the levels obtained with UCN or CRH (90.9 ± 11.0% relative to UCN; Fig. 5A), demonstrating that both CRHR1 and CRHR2 can contribute to the cAMP increases in αT3-1 cells.

To further confirm the contribution of CRHR2 to the UCN-mediated increases in cAMP, we utilized a selective CRHR2 antagonist, antisauvagine (Ruhmann et al. 1998, Brauns et al. 2002). This peptide has a 100- to 400-fold greater selectivity for CRHR2 than CRHR1. The cells were pretreated with antisauvagine for 15 min before the addition of UCN or the CRHR2-specific agonist, UCN 3. As shown in Fig. 5B, the cAMP levels after antisauvagine treatment alone did not significantly differ from the vehicle. However, antisauvagine completely blocked the UCN3-mediated increase in cAMP, while only partially blocking (75% repression) the UCN-mediated cAMP increases, consistent with both CRHR1 and CRHR2 contributing to the UCN-mediated cAMP increases.

UCN increases α-luciferase transcriptional activity

While LHB and FSHB are not produced in αT3-1 cells, the glycoprotein hormone α-subunit is actively transcribed and translated in these cells and its expression is highly regulated by GNRH1, gonadal steroids, and a variety of peptides including pituitary adenyl cyclase activating polypeptide (PACAP) and endothelin 1, working through numerous intracellular signaling pathways (reviewed in McArdle & Cousin (1996) and Evans (1999)). PACAP has been shown to increase the intracellular cAMP levels in αT3-1 cells, resulting in increased transcriptional activity and steady-state levels of α-subunit mRNA (Schomerus et al. 1994, Tsuji et al. 1995, Attardi & Winters 1998, Burrin et al. 1998). As the cAMP levels are also rapidly increased by CRH or UCN in these cells, we utilized a mouse α-subunit promoter–luciferase reporter construct (α-luciferase) in transfection studies to assess the increased transcriptional activity of the α-subunit promoter in αT3-1 cells after treatment with UCN. As shown in Fig. 6, UCN significantly increased α-luciferase activity in a dose-dependent manner from 30 to 300 nM, reaching levels that were not significantly different from those induced by forskolin, a direct activator of adenylyl cyclase. As the mouse α-subunit promoter is known to contain cAMP response elements and has previously been shown to be activated by increased cAMP/PKA activation...
In the rodent, Cshr1 mRNA has been localized primarily in the brain and intermediate and anterior lobes of the pituitary (Potter et al. 1994, Van Pett et al. 2000). While Cshr1 mRNA has previously been detected in a subset of rat corticotropes (Potter et al. 1994), recent studies from our laboratory have also demonstrated Cshr1 mRNA in a subset of cells expressing ACTH, prolactin, or LHB transcripts in the mouse anterior pituitary (Westphal et al. 2009), consistent with the expression of CRHR1 in the mouse gonadotrope-like αT3-1 cell line. CRHR2 expression in the rodent is quite distinct from CRHR1, with a more restricted pattern in the brain but numerous sites of expression in the periphery (Chalmers et al. 1996, Van Pett et al. 2000). While the two rodent isoforms of CRHR2, CRHR2α and CRHR2β, show comparable pharmacological properties (Hauger et al. 2006), they differ significantly in their tissue-specific expression. In situ hybridization studies using probes from the common region of CRHR2 have demonstrated Cshr2 mRNA expression in the posterior pituitary, with a weak but clearly detectable signal in the anterior pituitary (Van Pett et al. 2000), most likely in gonadotropes (Kageyama et al. 2003). CRHR2α mRNA has also been detected in anterior pituitary by ribonuclease protection assay (Kageyama et al. 2003). Consistent with these results, we have detected CRHR2α mRNA, but not CRHR2β, in αT3-1 cells by RT-PCR. Together, these results suggest that a population of gonadotropes may express both CRHR1 and CRHR2α at certain times in development or in response to specific hormonal signals.

The expression of CRHR1 and CRHR2α on αT3-1 cells that also express GNRH1 receptors is quite interesting in light of the recent studies demonstrating the significant population of anterior pituitary cells in rat and mouse that exhibit multiple hypothalamic releasing hormone (HRH) receptors (multi-responsive cells; Nunez et al. 2003, Villalobos et al. 2004). Recent studies have suggested that 38% of female mouse anterior pituitary cells express multiple HRH receptors, with almost 50% of LHB–positive cells being multi-responsive (Nunez et al. 2003). Our results are consistent with these studies and suggest that anterior pituitary cells are more multifunctional than previously thought, with great potential for crosstalk between HRH and pituitary hormonal release. Interestingly, CRH has recently been shown to induce thyrotropin release from the amphibian pituitary gland via CRHR2 signaling (Okada et al. 2007).

In response to treatment with CRH or UCN, the αT3-1 cells show a very rapid increase in the intracellular cAMP levels, demonstrating the positive coupling of the CRH receptors to Gs and adenyl cyclase after ligand stimulation. The rapid rise in the intracellular cAMP levels in αT3-1 cells in response to CRH or UCN with a peak at 2–5 min followed by a rapid decline is similar to the results seen with murine atrial cardiomyocyte tumor cells, AT-1 cells, which express endogenous CRHR2β mRNA (Heldwein et al. 1996). The rapid decline in the cAMP levels even in the

Schoderbek et al. 1992, Attardi & Winters 1998), these results are consistent with the CRH-mediated elevations in cAMP causing an increase in α-subunit promoter-directed transcriptional activity in αT3-1 cells.

### Discussion

We have demonstrated via RT-PCR that CRHR1 and CRHR2α mRNAs are expressed in αT3-1 cells. These G-protein-coupled receptors are functional and couple through Gs, as treatment of these cells with CRH or UCN results in significant increases in intracellular cAMP levels that can be blocked completely by the presence of non-selective CRH receptor antagonists. Our results also demonstrate that both CRHR1 and CRHR2α are functional in these cells as CRHR2-specific ligands increase cAMP levels to only 50–60% of the levels observed with non-selective agonists (CRH and UCN), and CRHR2-selective antagonists do not completely abolish the UCN-mediated increases in cAMP. In addition, we have shown that UCN treatment of αT3-1 cells results in increased transcriptional activity of the glycoprotein hormone α-subunit promoter, a promoter known to be positively regulated by elevated cAMP levels. These results are the first to demonstrate both the expression and functional signaling of CRHR1 and CRHR2α in murine gonadotrope-like cells, and they suggest a potential direct role for CRH or the other CRH-like ligands, UCN, UCN2, and UCN3, in the modulation of gonadotropin transcription and secretion at the level of the anterior pituitary gonadotrope. This proposed regulation at the pituitary could complement or modulate the CRH-mediated regulation of hypothalamic GNRH1 production and secretion.
presence of IBMX is quite interesting, and it may suggest that one or both types of CRH receptors on αT3-1 cells are desensitized quickly following exposure to ligand. This theory is supported by the reports of rapid agonist-induced CRHR1 desensitization and internalization (Roseboom et al. 2001, Telò et al. 2005). Alternatively, the dose of IBMX used in these studies may not be sufficient to inhibit all phosphodiesterase activity in these cells.

The responses to CRH and the urocortins in the αT3-1 cells were dose dependent and specific for CRH receptors, as the presence of non-selective CRH receptor antagonists, α-helical CRH 9–41 or AST, completely inhibited the intracellular cAMP increases. Similar peak cAMP values were observed with CRH or UCN, ligands that activate both CRHR1 and CRHR2α. By contrast, peak cAMP values with UCN2 and UCN3 stimulation reached levels only 50–60% of UCN- or CRH-stimulated levels, consistent with their CRHR2 selectivity. Similarly, the significant, but incomplete, inhibition of UCN-mediated increases in cAMP production in αT3-1 cells with antisauvagine, the CRHR2-specific antagonist (100- to 400-fold greater binding to CRHR2), was consistent with the UCN-mediated activation of both CRHR1 and CRHR2α. The EC50 values for cAMP production in αT3-1 cells were not significantly different between CRH, UCN, UCN2, or UCN3, and ranged from 14 to 32 nM. Studies in cell lines that have been stably or transiently transfected with CRH receptors have shown EC50 values for CRHR1 of 1–3 nM for both CRH and UCN and EC50 values for CRHR2 of 0.2–20 nM (Vaughan et al. 1995). Primary cultures or cultured cell lines expressing endogenous CRH receptors have shown higher EC50 values (Heldwein et al. 1996).

While stress-induced suppression of GNRH1 and LHB release is clearly dependent on central actions of CRH (Li et al. 2005, 2006), the finding that αT3-1 cells, a gonadotrope-like cell line, express CRH receptors suggests an additional and potential direct role for CRH or urocortins on gonadotrope function, consistent with the recent studies that have identified Crhr1 or Crhr2 mRNA in rat or mouse gonadotropes (Kageyama et al. 2003, Westphal et al. 2009). Our studies here extend the previous results to demonstrate that these CRH receptors are functional, increasing the intracellular cAMP levels resulting in transcriptional activation of cAMP-responsive genes including the glycoprotein hormone α-subunit. PACAP has also been shown to increase cAMP levels in αT3-1 cells, resulting in increased α-subunit expression (Schomerus et al. 1994, Tsuji et al. 1995, Attardi & Winters 1998). Interestingly, while GNRH1 does not alter cAMP levels in αT3-1 cells, GNRH1 significantly reduces the PACAP-mediated increase in cAMP levels via the activation of the PKC signaling pathway (McArdle et al. 1994, McArdle & Counis 1996), emphasizing the importance of crosstalk between distinct intracellular signaling pathways in these gonadotrope-like cells. It is possible that GNRH1 may also modify the CRH-mediated increases in cAMP in these cells. Similarly, we have initiated preliminary studies of GNRH1 signaling and phospholipase C (PLC) activation in αT3-1 cells. While GNRH1 treatment dramatically increases total inositol phosphate levels via PLC activation, CRH treatment alone has no significant effect on inositol phosphate levels (unpublished data, A. Seasholtz). However, if CRH and GNRH1 are co-administered, CRH significantly decreases the GNRH1-mediated increases in inositol phosphates, showing another level of crosstalk between the PKA and PLC/PKC signaling pathways in these gonadotrope-like cells (unpublished data, A. Seasholtz). Functional interactions between the PKA and PLC/PKC systems have also been observed in the LBT2 gonadotrope-like cell line, with GNRH1 stimulating PACAP receptor phosphorylation by PKC (Lariviere et al. 2008). Hence, while αT3-1 cells represent only a model of gonadotrope function, these studies suggest that CRH receptor signaling on pituitary gonadotropes may contribute, along with other peptide and gonadal hormones, to the modulation of GNRH1 signaling at these cells and resultant gonadotropin synthesis and release.

Finally, it should also be noted that the CRH-binding protein (CRHBP) is highly expressed in rodent anterior pituitary (Speert et al. 2002). This secreted glycoprotein binds CRH and UCN with high affinity and is thought to act largely as an inhibitory protein, binding the ligands and decreasing CRH receptor activation (reviewed in Westphal & Seasholtz (2006)). CRHBP expression is positively regulated by estrogen, resulting in a sexually dimorphic pattern of expression with CRHBP detected in corticotropes in male mice, while it is detected in corticotropes, lactotropes, and gonadotropes in female mice (Speert et al. 2002). CRHBP is also expressed in αT3-1 cells where its expression is regulated by GNRH1 (Westphal & Seasholtz 2005). Hence, the actions of CRH and UCN on αT3-1 cells or gonadotropes in vivo may be highly regulated by the pituitary CRHBP.

In conclusion, we have demonstrated that αT3-1 cells express Crhr1 and CRHR2α mRNAs and that these CRH receptors are functionally coupled to the Gs signal transduction pathways. These receptors respond to CRH, UCN, UCN2, or UCN3 treatment with rapid, transient increases in intracellular cAMP levels, and CRH receptor antagonist data confirm the contributions of both CRHR1 and CRHR2 to the cAMP inductions. The activation of these receptors also increases the transcriptional activity of the glycoprotein hormone α-subunit promoter, a well-characterized cAMP-responsive promoter. Since αT3-1 cells serve as a model for gonadotrope cells, these data suggest a potential direct role for CRH or the other CRH-like ligands, UCN, UCN2, and UCN3, in the coordinated control of gonadotropin expression and secretion at the gonadotrope. Studies in primary mouse anterior pituitary cultures and in vivo will allow us to further examine the actions of CRH and urocortins (and the CRHBP) on both basal and stimulated pituitary hormone release, potentially revealing new roles for these ligands in pituitary function.
Declaration of interest
The authors have no conflict of interest to report.

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