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

Audrey F Seasholtz^{1,2}, Miina Öhman³, Amale Wardani¹ and Robert C Thompson^{1,3}

¹Molecular and Behavioral Neuroscience Institute ²Department of Biological Chemistry and ³Department of Psychiatry, University of Michigan, 109 Zina Pitcher Place, BSRB Room 5035, Ann Arbor, Michigan 48109, USA

(Correspondence should be addressed to A F Seasholtz; Email: aseashol@umich.edu)

(M Öhman is now at Department of Internal Medicine, Cardiology, University of Michigan, Ann Arbor, Michigan, USA)

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 α T3-1 cells, a murine gonadotrope-like cell line. Reverse transcriptase PCR (RT-PCR) studies demonstrated that α T3-1 cells express mRNA for the two CRH receptor subtypes, CRHR1 and CRHR2, with CRHR2 α 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 α -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 α -subunit promoter as assessed by α -luciferase transfection assays. Together, these results demonstrate the functional signaling of CRH receptors in α T3-1 cells, suggesting that CRH may also modulate pituitary gonadotrope function *in vivo*.

Journal of Endocrinology (2009) **200**, 223–232

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-adenylyl 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 (CRHR2 α and CRHR2 β 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). CRHR2 α is expressed primarily in brain (Chalmers *et al.* 1995, Lovenberg *et al.* 1995a), while CRHR2 β is found largely in the periphery, but is also detected in the choroid plexus and cerebral arterioles (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 CRHR2 α 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 α T3-1 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

α T3-1 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

α T3-1 cells were plated (300 000 cells/well) onto 6-well plates (Falcon 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 isobutylmethylxanthine (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 μ l of 0.05 M Tris (pH 7.5), 4 mM EDTA, and cAMP levels were determined in 50 μ l 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 μ l/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 α T3-1 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-PCR

RT-PCR was performed in a final volume of 25 or 50 μ l using 1–3 μ l cDNA product, 200 μ M deoxyribonucleotides, 1.0 μ M primers, *Taq* buffer with $MgCl_2$, 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-GCA-GTG-TGA-GAG-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'-CTC-CTC-AGC-CTG-CTG-GAG-GCC-AAC-T-3' and the reverse primer was 5'-GTT-CTC-CAG-GCA-CTC-TCT-GTA-GGC-ATT-3' (exons 3–5, 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'-GGC-CTA-AGA-GAG-AGG-CCG-GAC-AGA-CCT-CCT-TTG-GA-3' and the reverse primer was 5'-AGA-ATG-AAG-GTG-GTG-ATG-AGG-TTC-CAG-TGG-ATC-ACA-3' (exons 1–7, amplifying a 631 bp fragment from nucleotides 40 to 670 of mouse CRHR2 β cDNA, GenBank accession no. NM_009953). Both PCR amplifications included 30 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.

Transient transfections and reporter constructs

Cultures of α T3-1 cells were plated onto 6-well plates (400 000 cells/well) in DMEM + 10% FCS + gentamicin (50 μ g/ml). The cells were transfected 12–24 h later, using FuGENE (see the manufacturer's recommendations; Roche) with 1.75 μ g luciferase reporter DNA and 0.25 μ g RSV- β gal 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- β gal was used to normalize for transfection efficiency. For all studies, 10 μ M 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 $\times$ PBS, pelleted, and lysed in 100 μ l lysis buffer (0.25 M Tris (pH 8.0), 0.1 M EDTA, 15 mM $MgSO_4$, 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 μ l of each supernatant were added to 100 μ l 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 $\pm$ 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 α T3-1 cells

RT-PCR was utilized to characterize the expression of *Crhr1* and *Crhr2* mRNA in α T3-1 cells. cDNA synthesis reactions

Figure 1 Expression of CRHR1 and CRHR2 α RNA in α T3-1 cells. RT-PCR was utilized to test for the expression of CRH receptor RNA in α T3-1 cells. (A) Utilizes primers specific for CRHR1 and (B) and (C) utilize primers specific for CRHR2 α and CRHR2 β respectively. The presence or absence of reverse transcriptase (RT) during cDNA synthesis is indicated by + or – signs. (A) An amplified product of 394 bp in hypothalamus and α T3-1 cDNA lanes (see arrow). Hy+ (hypothalamus, with RT), Hy– (hypothalamus, without RT), α T+ (α T3-1, with RT), α T– (α T3-1, without RT), W (water control). (B) An amplified product of 243 bp in α T3-1 (α T+) and brain (Br+) cDNA lanes (see arrow). These bands are consistent with the expression of CRHR2 α in α T3-1 and total brain RNA samples. (C) Amplified products of 631 bp only in the brain (Br+) lane (see arrow). The absence of amplified product in the α T+ lane indicates that CRHR2 β is not detectable in α T3-1 RNA. M represents 1 kb plus (Invitrogen) DNA ladder in (A) and 1 kb (Invitrogen) DNA ladder in (B) and (C).

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 α T3-1 cells (α T+) 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 α T3-1 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: CRHR2 α and CRHR2 β (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 CRHR2 α or CRHR2 β mRNAs. The results shown in Fig. 1B demonstrate the presence of an appropriately sized fragment for CRHR2 α (243 bp) in α T3-1 (α T+) and mouse total brain (Br+) cDNA. The 243 bp fragment from α T3-1 cDNA was subcloned; DNA sequence analysis confirmed that the fragment contained the CRHR2 α cDNA sequence. Additional primer pairs also confirmed the presence of CRHR2 α in α T3-1 cells (data not shown). The cDNA samples used for the PCR in Fig. 1B were also tested with several different CRHR2 β primer pairs. While the 631 bp CRHR2 β -specific PCR product is readily detected in total brain (Br+) (Fig. 1C) or mouse heart (not shown), CRHR2 β is not detected in the same α T3-1 cDNA samples (α T+) that readily detected CRHR2 α . Additional primer pairs also failed to demonstrate detectable levels of CRHR2 β in the α T3-1 samples. Together, the data in Fig. 1A–C clearly demonstrate the presence of *Crhr1* and CRHR2 α mRNAs in α T3-1 cells, with non-detectable levels of CRHR2 β transcripts.

Stimulation of cAMP production in α T3-1 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 α T3-1 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 α T3-1 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 α T3-1 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

Figure 2 Time-dependent increases in intracellular cAMP levels in α T3-1 cells after treatment with CRH or UCN. The cells were treated with 200 nM CRH or UCN for 2, 5, 10, 20, and 40 min in the presence of 1 mM IBMX. cAMP assays were performed on cell lysates. The addition of CRH ($\square$) or UCN ($\circ$) resulted in a rapid increase in intracellular cAMP levels when compared with vehicle treatment (dashed line). Points represent the average ($\pm$ S.E.M.) for triplicate wells from a representative experiment.

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 α T3-1 cells 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 (EC_{50}) concentrations of CRH and UCN were not significantly different with the values of $14.5 \text{ nM} \pm 5.9$ ($n=3$) and $21.2 \text{ nM} \pm 5.1$ ($n=7$) 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 AST) that block both CRHR1 and CRHR2 (reviewed in Hauger *et al.* (2006)) to determine whether the CRH- and

Figure 3 Dose-dependent increases in cAMP levels in α T3-1 cells following treatment by CRH ($\square$) or UCN (Δ). Intracellular levels of cAMP are shown after the treatment of α T3-1 cells with various concentrations of CRH or UCN. Treatments were for 3 min. Points are the average ($\pm$ S.E.M.) from duplicate assays on triplicate wells from a representative experiment. The maximal cAMP levels are not significantly different following CRH or UCN treatment and the EC_{50} values are also not significantly different.

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 μ M α -helical CRH 9-41 (9-41, Fig. 4A) or 1 μ M 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

Figure 4 Inhibition of CRH- or UCN-mediated stimulation of cAMP levels by CRH receptor antagonists α -helical CRH 9-41 or astressin. (A) α T3-1 cells were treated with DMEM/IBMX containing vehicle, 10 μ M α -helical CRH 9-41 (9-41), 100 nM CRH, 100 nM UCN, or 9-41 plus CRH or UCN for 3 min. Intracellular cAMP levels after 9-41, CRH, or UCN treatments were significantly increased from vehicle-treated levels (VEH; *significance from VEH, $P<0.05$). In addition, treatments with 9-41 in the presence of CRH or UCN blocked the CRH- and UCN-mediated increases in intracellular cAMP (#significantly different from CRH or UCN treatments alone, $P<0.05$). (B) α T3-1 cells were treated as above except that astressin (AST, 1 μ M) was used alone or in combination with 100 nM CRH or UCN. These results also show significant inductions in cAMP levels with CRH and UCN and significant repressions of CRH- or UCN-mediated increases in cAMP by the addition of astressin. * and # are used as in (A).

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 α T3-1 cells

To determine the contribution of CRHR2 to the overall cAMP increases in α T3-1 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 $20.3 \text{ nM} \pm 9.7$ ($n=3$) and $32.5 \text{ nM} \pm 8.2$ ($n=3$), not significantly different from each other or the EC₅₀ value of UCN ($21.2 \pm 5.2 \text{ nM}$, $n=7$). At maximal doses of UCN2 and UCN3 (100 nM), we observed increases in cAMP levels that reached $60.0 \pm 7.6\%$ ($n=6$) and $54.3 \pm 6.0\%$ ($n=13$) of the levels obtained with UCN or CRH ($90.9 \pm 11.0\%$ relative to UCN; Fig. 5A), demonstrating that both CRHR1 and CRHR2 can contribute to the cAMP increases in α T3-1 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 α T3-1 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 adenylyl cyclase activating polypeptide (PACAP) and endothelin 1, working through numerous intracellular signaling pathways (reviewed in McArdle & Counis (1996) and Evans (1999)). PACAP has been shown to increase the intracellular cAMP levels in α T3-1 cells, resulting in increased transcriptional activity and steady-state levels of α -subunit mRNA (Schomerus *et al.* 1994, Tsujii *et al.* 1995, Attardi & Winters 1998, Burrin *et al.* 1998). As the cAMP levels are also rapidly increased by CRH or UCN

Figure 5 Functional role of CRHR2 in cAMP signaling in α T3-1 cells. (A) α T3-1 cells were treated with vehicle, CRH, UCN, UCN2, or UCN3 (100 nM) for 3 min. Intracellular cAMP levels were determined and the increase in cAMP levels under each treatment condition (from VEH) was normalized to that observed with UCN (set to 100%). UCN2 and UCN3 showed significantly lower elevations in cAMP, showing only 50–60% of the increases obtained with UCN or CRH (*significantly different from UCN, $P<0.05$), suggesting that CRHR2 contributes ~50% to the cAMP increases observed after stimulation with UCN. (B) α T3-1 cells were pretreated with antisauvagine (AS, 1 μ M) or vehicle for 15 min followed by a 3-minute treatment with UCN or UCN3 (100 nM) in the presence or absence of antisauvagine. Intracellular cAMP levels after UCN treatments were significantly increased from vehicle-treated levels (VEH; *significantly different from VEH, $P<0.05$), with UCN-mediated increases being significantly greater than UCN3-mediated increases. There was no significant difference in the intracellular cAMP levels between VEH and AS treatments. Treatment with AS in the presence of UCN3 completely blocked the UCN3-mediated increases (^bsignificantly different from UCN3, $P<0.05$), resulting in cAMP levels not significantly different from VEH or AS. Treatment with AS in the presence of UCN significantly reduced cAMP levels from UCN (^asignificantly different from UCN, $P<0.05$), but cAMP levels remained significantly greater than VEH, AS/VEH, or AS/UCN3 levels, suggesting that activated CRHR1 was responsible for the remaining cAMP increase.

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 α T3-1 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

Figure 6 Dose-dependent increases in α -luciferase activity in transiently transfected α T3-1 cells following treatment with UCN. Treatment of α -luciferase-transfected α T3-1 cells with UCN (30 nM, 100 nM, or 300 nM, 4 h) or forskolin (10 μ M, 4 h) significantly induced α -luciferase promoter activity. The relative promoter activity is represented as fold induction over control. Values represent the mean $\pm$ S.E.M. ($n=3$). * $P<0.05$, when compared with vehicle control.

(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 α T3-1 cells.

Discussion

We have demonstrated via RT-PCR that *CRHR1* and *CRHR2 α* mRNAs are expressed in α T3-1 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 *CRHR2 α* 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 α T3-1 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 *CRHR2 α* 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.

In the rodent, *Crhr1* 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 *Crhr1* mRNA had previously been detected in a subset of rat corticotropes (Potter *et al.* 1994), recent studies from our laboratory have also demonstrated *Crhr1* 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 α T3-1 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*, *CRHR2 α* and *CRHR2 β* , 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 *Crhr2* 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). *CRHR2 α* mRNA has also been detected in anterior pituitary by ribonuclease protection assay (Kageyama *et al.* 2003). Consistent with these results, we have detected *CRHR2 α* mRNA, but not *CRHR2 β* , in α T3-1 cells by RT-PCR. Together, these results suggest that a population of gonadotropes may express both *CRHR1* and *CRHR2 α* at certain times in development or in response to specific hormonal signals.

The expression of *CRHR1* and *CRHR2 α* on α T3-1 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 α T3-1 cells show a very rapid increase in the intracellular cAMP levels, demonstrating the positive coupling of the CRH receptors to Gs and adenylyl cyclase after ligand stimulation. The rapid rise in the intracellular cAMP levels in α T3-1 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 *CRHR2 β* mRNA (Heldwein *et al.* 1996). The rapid decline in the cAMP levels even in the

presence of IBMX is quite interesting, and it may suggest that one or both types of CRH receptors on α T3-1 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, Teli *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 α T3-1 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 CRHR2 α . 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 α T3-1 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 CRHR2 α . The EC₅₀ values for cAMP production in α T3-1 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 α T3-1 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 α T3-1 cells, resulting in increased α -subunit expression (Schomerus *et al.* 1994, Tsujii *et al.* 1995, Attardi & Winters 1998). Interestingly, while GNRH1 does not alter cAMP levels in α T3-1 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 α T3-1 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 L β T2 gonadotrope-like cell line, with GNRH1 stimulating PACAP receptor phosphorylation by PKC (Lariviere *et al.* 2008). Hence, while α T3-1 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 α T3-1 cells where its expression is regulated by GNRH1 (Westphal & Seasholtz 2005). Hence, the actions of CRH and UCN on α T3-1 cells or gonadotropes *in vivo* may be highly regulated by the pituitary CRHBP.

In conclusion, we have demonstrated that α T3-1 cells express *Crhr1* and *CRHR2 α* 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 α T3-1 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.

Funding

The studies were supported by NIH grants DK54323 to R C T and DK42730 and DK57660 to A F S. We gratefully acknowledge the postdoctoral support of M Öhman from the Academy of Finland (grant no. 52840) and the Finnish Medical Foundation.

Acknowledgements

We would like to thank Linda Harper Gates and Jyoti Kothavale for their assistance with α T3-1 studies. This work was presented in part at the 85th Annual Meeting of the Endocrine Society, Philadelphia, PA. While this manuscript was under revision, a separate manuscript from our laboratory was published, which documented CRH receptor expression in multiple anterior pituitary cells lines (including α T3-1 cells) and in murine corticotropes, lactotropes, and gonadotropes (Westphal *et al.* 2009).

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Received in final form 22 October 2008

Accepted 11 November 2008

Made available online as an Accepted Preprint
13 November 2008