Expression and signalling characteristics of the corticotrophin-releasing hormone receptors during the implantation phase in the human endometrium

E Karteris, N Papadopoulou, D K Grammatopoulos and E W Hillhouse¹

Molecular Medicine, Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, UK

¹The Medical School, University of Leeds, Leeds LS2 9NL, UK

(Requests for offprints should be addressed to E Karteris; Email: ekarteris@bio.warwick.ac.uk)

Abstract

Corticotrophin-releasing hormone (CRH) has been identified in several peripheral tissues, including the female reproductive organs. CRH is expressed in the placenta, myometrium, epithelial endometrium and the endometrial stromal cells at all phases of the menstrual cycle. Similarly, CRH receptors are present in pregnant and non-pregnant myometrium, placenta and endometrium. Putative roles of CRH in the endometrium include involvement in implantation, decidualisation and maintenance of pregnancy. In this study we sought to investigate in detail the CRH receptor repertoire expressed in the human endometrium and their signalling characteristics. Using RT-PCR we were able to demonstrate the expression of CRH receptor 1 α (CRH-R1 α) and CRH-R2 α in the human endometrium. CRH-R1 β was present in 40% of endometrial cDNAs examined. No apparent expression of CRH-R2 β , CRH-R2 γ or any other CRH-R1 splice variants was detected. Chemical cross-linking studies with ¹²⁵I-ovine CRH revealed that the endometrial CRH receptor has a molecular weight of 45 kDa. Using the non-hydrolysable photoreactive analogue [α -³²P]GTP-azidoanilide and peptide antisera raised against G-protein α -subunits, we then studied coupling of endometrial CRH receptors to G proteins. Treatment of endometrial membranes with human CRH (100 nM) increased the labelling of Gq and Gs, but not Gi or Go. These results were supported by experiments in epithelial cells of the non-pregnant human endometrium in the secretory phase which showed that CRH induced increases in both cAMP and inositol trisphosphate levels. These results suggested that CRH may exert multiple effects in the human endometrium via distinct signalling cascades. These events are possibly mediated via different receptor subtypes.

Journal of Molecular Endocrinology (2004) **32**, 21–32

Introduction

Since its discovery, it has become evident that the physiological role of corticotrophin-releasing hormone (CRH) is much wider than initially thought. In the female reproductive system, CRH and its receptors have been identified in the human placenta, fetal membranes, ovaries (Petraglia *et al.* 1987, Riley *et al.* 1991, Mastorakos *et al.* 1994, Hatzoglou *et al.* 1996, Asakura *et al.* 1997, Karteris *et al.* 1998, 2000), and in the pregnant and non-pregnant myometrium where CRH is thought to play an important role in the mechanism of labour (Hillhouse *et al.* 1993, Grammatopoulos & Hillhouse 1999). In addition,

maternal decidua synthesises and releases CRH and there is an increase in decidual CRH mRNA expression during gestation (Petraglia *et al.* 1992).

CRH has also been detected both *in vivo* and *in vitro* in normal and neoplastic human endometrial epithelial cells (Makriganakis *et al.* 1995a). In addition, CRH and its type 1 CRH receptor (CRH-R1) have been detected in human endometrial stromal cells in both the proliferative and secretory phase, the latter involving the decidualisation of stroma (Di Blasio *et al.* 1997). Recently, the CRH-R1 receptor has also been detected in the human endometrial adenocarcinoma Ishikawa cell line (Graziani *et al.* 2002).

In vitro studies of human endometrial cells indicate that CRH can induce decidualisation of stromal cells (Ferrari *et al.* 1995) and can also stimulate the production of interleukin (IL)-1 and IL-6 (Zoumakis *et al.* 2000). It has been hypothesised that endometrial CRH may participate in the initiation of local inflammatory events leading to the formation of egg niddus (Gravanis *et al.* 2001). Indeed, studies have shown that both epithelial and decidualised stromal cells of the early pregnant rat uterus contain immunoreactive CRH and that the content of CRH mRNA and its peptide product is higher in implantation sites of early pregnant rat uterus (Makrigiannakis *et al.* 1995b).

In rodents, immunoneutralisation of CRH or blockage of the CRH-R1 receptor site with the antagonist antalarmin decreases the number of live embryos (Athanasakis *et al.* 1999, Makrigiannakis *et al.* 2001). The mechanism whereby CRH promotes blastocyst implantation and early maternal tolerance appears to be via Fas ligand (FasL) expression in human extravillous trophoblasts with an invasive phenotype (Makrigiannakis *et al.* 2001). This is of increasing importance, since the Fas receptor and its ligand play an important role in the regulation of immune tolerance (Makrigiannakis *et al.* 2003). The major function of the Fas–FasL interaction is the induction of apoptosis (Nagata 1994), which is one of the key events involved in the implantation of the blastocyst into the receptive endometrium (Selam *et al.* 2001).

It appears therefore that endometrial CRH is a potent modulator of blastocyst implantation. The purpose of this study was to investigate in detail the expression, G-protein coupling and signal transduction pathways of CRH receptors during the implantation phase in the human endometrium.

Materials and methods

Subjects

Endometrial biopsy samples ($n=8$) were obtained from normal fertile women undergoing diagnostic hysteroscopy or laparoscopy for pelvic pain. They were performed on the day corresponding to luteinizing hormone (LH)+6 to LH+8 of the cycle. Chronological dating was based on the last menstrual period and histological dating was performed according to the criteria established by Noyers *et al.* (1950). Women were defined as being

normally fertile if they had one or more successful pregnancies, a regular menstrual cycle (25–35 days) and had not received any steroid hormone or an intrauterine contraceptive device for 3 months prior to the collection of the biopsy sample. Their ages ranged from 27 to 39 years. Ethical approval was obtained from the local ethical committee, and informed consent was obtained from each patient before inclusion in this study.

Cell culture

Primary cell cultures were prepared from endometrial biopsy sample collected in Hank's balanced salt solution containing streptomycin and penicillin (100 µg/ml). The tissue was chopped finely and incubated at 37 °C in 5 ml Dulbecco's modified Eagle's medium (DMEM) containing 0.2% collagenase (type 1A). After 60 min of digestion, filtration through a 250 µm sieve (Nylon Bolting Cloth; Lockortex, Warrington, Cheshire, UK) was carried out to separate the undigested pieces of tissue and the mucous material from the cells, followed by a second filtration through a 40 µm sieve to separate the epithelial cells (predominantly present as glands) from the stromal and red blood cells (single cells) (Fernandez-Shaw *et al.* 1992). The single cells were collected by centrifugation at 300 *g* and the pellet was resuspended in culture medium (DMEM containing 10% fetal calf serum, 4 mM glutamine and antibiotics). The glands were backwashed from the filter with phosphate-buffered saline (PBS), digested for a further 30 min as before, collected by centrifugation at 100 *g* and resuspended in culture medium. The purified epithelial cells were grown until confluence in two-chamber glass slide flaskettes (Nunc, Inc., Naperville, IL, USA) and fixed in methanol/acetone (50:50) for 90 s.

Fluorescent *in situ* hybridisation (FISH)

Fixed endometrial epithelial cells were dehydrated by successive washes through ethanol and air-dried. A specific 40-mer synthetic oligonucleotide probe for the CRH-R1 with fluorescein conjugated at their 5'-ends was used in this study (Fluo-CCA CTAGCTGCCCCGCAGGGCTGCGGGGCCA GCAGGTGCC). Hybridisation solution (100 µl) containing 1 ng/µl of the probe, was allowed to hybridise at 37 °C overnight. Slides were then

Table 1 List of primers used for the amplification of CRH receptor subtypes in human endometrium

Subtype	Primer	
CRH-R1 α / β	Sense	5'-GGCAGCTAGTGGTTCGGCC-3'
	Antisense	5'-TCGCAGGCACCGGATGCTC-3'
CRH-R1c	Sense	5'-ACATCTCAGACAATGGCTAC-3'
	Antisense	5'-TCGCAGGCACCGGATGCTC-3'
CRH-R1 α /d	Sense	5'-CCGCATCCTCATGACCAAGC-3'
	Antisense	5'-CTCTTCCGGATGGCAGAACG-3'
CRH-R2 α	Sense	5'-AAGGTCCCCACTTGACAG-3'
	Antisense	5'-TAGGGACCCTCGGGGTCCAG-3'
CRH-R2 β	Sense	5'-CCCTCACCAACCTCTCAGGTCC-3'
	Antisense	5'-CAGGTCATACTTCCTCTGCTTGTC-3'
CRH-R2 γ	Sense	5'-CTCAAGCAATCTGCCTACCT-3'
	Antisense	5'-GGCTCACACTGTGAGTAGTT-3'
β -actin	Sense	5'-AAGAGAGGCATCCTCACCCT-3'
	Antisense	5'-TACATGGCTGGGGTGTGAA-3'

placed in preheated (45 °C) 2 × SSC buffer, in which they were washed twice, followed by another 10-min immersion in 0.1 × SSC (45 °C). The tissue sections were rinsed with PBS, and the cell nuclei were visualised by applying the DNA-specific dye 4,6-diamido-2-phenylindole (DAPI) at a final concentration of 1 µg/ml.

Immunofluorescence

Fixed endometrial epithelial cells were washed in PBS and incubated with 3% bovine serum albumin (BSA) for 1 h before incubation with the first primary goat polyclonal CRH-R1/2 cross-reactive antibody for 60 min, which was used at 2 µg/ml. All dilutions were made in 3% BSA in PBS. After three washes with PBS, specimens were incubated for 30 min with the second primary mouse monoclonal cytokeratin antibody followed by another set of washes, as before. Incubation with the first secondary anti-goat IgG–fluorescein isothiocyanate (FITC) conjugate was carried out for 2 h in the dark, followed by three washes with PBS and addition of the second secondary anti-mouse IgG–tetramethylrhodamine isothiocyanate (TRITC) conjugated antibody for 30 min. Specimens were washed thoroughly and before mounting the coverslips using 90% glycerol-PBS, the nuclei were stained with DAPI at a final concentration of 1 µg/ml. The results were viewed under fluorescent microscope using appropriate filters.

Total RNA extraction and cDNA synthesis

Total RNA was prepared from individual samples using RNeasyTM Total RNA Kit (QIAGEN, Crawley, East Sussex, UK) according to the manufacturer's guidelines. First-strand cDNA synthesis was performed using RNase reverse transcriptase (GIBCO BRL, Paisley, Strathclyde, UK).

PCR

All PCR reactions were carried out using Taq DNA polymerase (GIBCO BRL) with 200 ng cDNA for each amplification, as previously described (Karteris *et al.* 2001b). Briefly, endometrial cDNAs were amplified at 94 °C (45 s), 58 °C (45 s), 72 °C (1 min), in a total of 30 cycles with a final extension step at 72 °C for 10 min. The set of primers for the amplification of the CRH receptor subtypes and β -actin are shown in Table 1. Ten microlitres of the reaction mixture were subsequently electrophoresed on a 1.6% agarose gel and visualised by ethidium bromide, using a 1 kb DNA ladder (GIBCO BRL) to estimate the band sizes. As a negative control for all of the reactions, distilled water was used in place of the cDNA. The resultant PCR products were sequenced in an automated DNA sequencer and the sequence data were analysed using Blast Nucleic Acid Database Searches from the National Centre for Biotechnology Information.

Table 2 G-protein antisera used in this study. All antisera were raised in rabbits

Antibody	Size (kDa)	α -subunit	Sequence	Binding site
Gs	47.45	α s	RMHLRQYELL	C-terminus
Go	40.5	α o	GCTLSAEERAALERSK	N-terminus
Gi	41	α i _{1/2}	KENLKDCGLF	C-terminus
Gq	42	α q/11	QLNLKEYNLV	C-terminus

Preparation of endometrial membranes

Tissues were weighed and homogenised in 6 ml Dulbecco's PBS containing 10 mM MgCl₂, 2 mM EGTA, 0.15% BSA (w/v), 0.15 mM bacitracin and 1 mM phenylmethylsulfonyl fluoride, pH 7.2 (extraction buffer) at 22 °C for 40 s. The homogenate was centrifuged at 3000 r.p.m. for 30 min at 4 °C. The resultant pellet was washed, resuspended in extraction buffer, and spun at 19 000 r.p.m. for a further 60 min at 4 °C. The final pellet was resuspended in extraction buffer using homogeniser for 20 s.

Binding studies

Endometrial membrane suspensions (100 µg) were added to polypropylene tubes with 50 µl ¹²⁵I-ovine (o) CRH and ¹²⁵I-urocortin (UCN; 50 000 c.p.m.) and 50 µl extraction buffer or unlabelled peptides (diluted in buffer). The unrelated peptide arginine vasopressin (AVP) was used as a control. The tubes were incubated at 22 °C for 2 h after which the reaction was terminated by the addition of 1 ml ice-cold polyethylene glycol (20% w/v). The tubes were spun at 3000 r.p.m. for 30 min at 4 °C. The supernatant was discarded and the membrane-bound radioactivity present in the pellet was measured in a gamma counter.

Chemical cross-linking and SDS-PAGE

Human endometrial membranes were incubated with ¹²⁵I-oCRH for 2 h in 300 µl buffer (50 mM Tris-HCl, 2 mM EGTA, 10 mM MgCl₂ and 1.5 g/l BSA, pH 7.2) at 22 °C to reach equilibrium in the presence or absence of cold CRH (100 nM) to define non-specific binding. Disuccinimidyl suberate (10 µl) was added to the preparation to give a final concentration of 1.5 mM. The reaction

was then allowed to proceed for 10 min at 22 °C before termination with 1 ml ice-cold extraction buffer and centrifugation at 12 000 r.p.m. for 10 min. The pellets were then washed, solubilised and subjected to SDS-PAGE as previously described (Grammatopoulos *et al.* 1995). The gel was then dried and exposed to Fuji X-ray film at – 70 °C for 3 days.

Synthesis of [α -³²P]GTP-azidoanilide (GTP-AA) and photoaffinity labelling of α -subunits

GTP-AA was synthesised using a method previously described (Grammatopoulos *et al.* 1999). Human endometrial membranes (150–200 µg) were incubated for 3 min at 30 °C with CRH (100 nM) in buffer C (50 mM HEPES, 30 mM KCl, 10 mM MgCl₂, 1 mM benzamidine and 0.1 mM EDTA), followed by the addition of 5 µM GDP and 6 µCi GTP-AA. After incubation for 3 min at 30 °C in a darkened room, membranes were placed on ice and collected by centrifugation at 13 000 r.p.m. for 15 min at 4 °C. The supernatant was carefully removed, and the membrane pellet was resuspended in 120 µl buffer C containing 1.6 mg/5 ml dithiothreitol (DTT). Samples were vortexed and irradiated for 5–10 min at 4 °C with an ultraviolet light (254 nm) from a distance of 5 cm, to cross-link the GTP-AA to the G proteins. Immunoprecipitation using 10 µl undiluted G-protein antisera (Table 2) was then carried out as previously described (Grammatopoulos *et al.* 1999).

cAMP studies

Endometrial cell membrane preparations (50 µg protein) were preincubated with different concentrations of human (h)/rat (r) CRH (0.1–1000 nM), in 50 µl extraction buffer for 30 min at 22 °C, prior

to the addition of 100 µl 50 mM Tris-HCl containing 10 mM MgCl₂, 1 mM EGTA, 1 g/l BSA, 1 mM ATP, ATP regeneration system (7.4 mg/ml creatine phosphate, 1 mg/ml creatine phosphokinase), 100 µM 3-isobutyl-1-methylxanthine and 0.15 mM bacitracin, pH 7.4 at 37 °C. The reaction was terminated after 10 min by the addition of 1 ml 0.1 M imidazole buffer, pH 7, followed by heating of the tubes in boiling water for 5 min. The amount of cAMP in the supernatants was determined by radioimmunoassay.

Standard cAMP concentrations, covering the range 0.138–100 pmol/ml, were used for determination of the standard curve of the radioimmunoassay. The interassay coefficient of variation was 8% cAMP assay buffer (without any membrane preparations) was used as the negative control.

Inositol trisphosphate (IP₃) assay

For the IP₃ assay, endometrial cell membrane suspensions were incubated with increasing concentrations of CRH, followed by the addition of 200 µl IP₃ generation buffer containing 25 mM Tris-acetate buffer, pH 7.2, 5 mM Mg acetate, 1 mM DTT, 0.5 mM ATP, 0.1 mM CaCl₂, 0.1 mg/ml BSA and 10 µM GTP. Membranes were incubated for 3 min at 37 °C, and the reaction was terminated by the addition of 1 M ice-cold trichloroacetic acid, followed by extraction of inositol phosphates and neutralisation. IP₃ levels were estimated by radioimmunoassay based on the displacement of ³H[IP₃] from a specific bovine adrenocortical IP₃ binding protein. The interassay coefficient of variation was 11.7%.

Statistical analysis

Data are shown as the mean ± S.E.M. of each measurement. For the second messenger measurements, a one-way analysis of variance was used, followed by Dunnett's test, in order to compare each treatment dose of CRH with the basal condition. The relative density of the bands was measured by optical density scanning using the software Scion Image-Beta 3b for Windows (Scion Corporation, Frederick, MD, USA). In each case, results were evaluated between groups by using two-tailed Student's *t*-test, with significance determined at the level of *P* < 0.05.

Results

Characterisation of cell cultures

Human endometrial epithelial cell cultures were grown in a monolayer, forming whirl-like structures in the first 2 days and a confluent monolayer by day 5. The method used for separating glands from stromal cells resulted in a 1–2% contamination of stromal cells in the epithelial cell cultures, as confirmed by immunocytochemical analysis using anti-vimentin (fibroblast cell marker) and anti-cytokeratin (epithelial cell marker) (data not shown).

FISH

FISH was carried out in order to investigate the expression and localisation of CRH-R1 mRNA in the human endometrial epithelial cells. A specific probe for the CRH-R1 receptor was designed from the N-terminus region of the receptor which recognises the CRH receptor subtypes 1α and 1β, but not any of the CRH-R2 subtypes. As shown in Fig. 1A, the expression of CRH-R1 receptor mRNA is demonstrated as positive fluorescein staining localised in the cytoplasm of the cells. The cells were also viewed under Hoffman augmented phase contrast, which enabled us to confirm their type by the characteristic epitheloid morphology of endometrial epithelial cells in culture (Fig. 1B). No staining was detectable in endometrial cells probed with sense oligonucleotide probe for the CRH-R1 (Fig. 1C and D). Similar findings were observed when a fluo-probe for the CRH-R2 was utilised (data not shown). In mixed cultures, CRH receptor expression was barely detectable in stromal cells *in vitro* (data not shown). This might be due to tissue manipulation upon preparation of primary cell cultures, since studies on human myometrial cells have shown loss of CRH receptors as the number of passages of primary cultures increases (Linton *et al.* 2001, Aggelidou *et al.* 2002).

Immunofluorescence

In order to investigate the CRH receptor protein expression in the endometrial epithelial cell cultures, immunofluorescence was carried out using CRH-R1/2 specific antibody. A plasma membrane rich in positive staining granules was revealed (Fig. 1E). To confirm the type of endometrial cells

that were immunopositive for the receptor, double staining was performed on the same specimens for CRH-R1/2 and cytokeratin (epithelial cell marker). As shown in Fig. 1F, the endometrial cells were positive for cytokeratin. No staining was detected in the untransfected HEK-293 cells, which were used as negative controls for CRH receptors (Fig. 1G and H). These results demonstrated that the human endometrial epithelial cells express the CRH receptor protein.

Furthermore, in agreement with our FISH studies, we were unable to detect any staining for CRH receptors in stromal cells *in vitro* (data not shown).

PCR analysis

Using primers which reverse transcribe either CRH-R1 α or CRH-R1 β receptor subtypes we were able to detect CRH-R1 α in endometrial biopsy samples and epithelial cells. However, only 40% of the cDNAs encoded CRH-R1 β mRNA (Fig. 2A). We were unable to detect the CRH-R1c and CRH-R1d splice variants in this tissue (data not shown). PCR amplification using specific primers for the CRH-R2 receptor subtypes resulted in the detection only of CRH-R2 α in the human endometrium (Fig. 2A). None of the other type-2 receptor subtypes (CRH-R2 β and CRH-R2 γ) were detected. Sequence analysis confirmed the identity of all of the PCR products.

Displacement and chemical cross-linking studies

The presence of functional CRH receptors in endometrial tissue was confirmed by binding displacement studies. h/rCRH and UCN were able to displace 125 I-oCRH and 125 I-UCN respectively from its binding sites in a concentration-dependent manner in endometrial membranes (Fig. 2B). The

specificity of the receptor was assessed by co-incubating 125 I-oCRH with the unrelated peptide AVP (at concentrations up to 100 nM) which was unable to displace 125 I-oCRH and 125 I-UCN (data not shown). Using chemical cross-linking, we have also shown that there was a specific CRH-binding site with an apparent molecular mass of 45 kDa (Fig. 2C).

G-protein activation and downstream second messenger generation

Activation of G α and adenylate cyclase system by CRH

In order to identify the G proteins activated by CRH, we use the non-hydrolysable GTP analogue [α - 32 P]GTP-AA which binds – upon receptor stimulation – to the GTP-binding site of activated G α proteins. Immunoprecipitation with specific G α chain antibodies was used to identify agonist-dependent activation of G proteins (Offermanns *et al.* 1991).

Our results indicated that treatment of endometrial membranes with CRH (100 nM) increased the labelling of Gq and Gs, and but not Gi or Go which are present in human endometrium (Bernardini *et al.* 1995). Interestingly, both short forms of Gs α subunits (47 and 45 kDa) were activated by CRH receptors. CRH increased the incorporation of GTP-AA preferentially to the 47 (2.8 ± 0.2 -fold above basal) rather the 45 kDa isoform (1.6 ± 0.1 -fold above basal). This differential activation of the short Gs isoforms appeared to be significant ($P < 0.05$) (Fig. 3A, insert), in all samples tested ($n=4$).

To test the ability of CRH to activate endometrial epithelial cell adenylate cyclase, we determined the effect of CRH on cAMP production. When membranes from epithelial cells were incubated with CRH (10 pM to 100 nM) for 30 min at 25 °C, there was a significant increase in cAMP production. This increase was found to be

Figure 1 FISH and immunostaining showing the expression of the CRH-R1 in epithelial cells of human endometrium. (A) CRH-R1 mRNA expression is shown as fluorescein staining in the cytoplasm of the epithelial endometrial cells; (B) the same field viewed as Hoffman augmented phase contrast. Note the characteristic epitheloid morphology of the cells in culture. (C) No staining was obtained in the endometrial cells hybridised with sense oligonucleotide probe for CRH-R1; (D) the nuclei of the same cells are shown with DAPI staining. (E) CRH-R1 protein detected by immunostaining as fluorescent granules on the cell membrane; (F) double staining of the same cells confirms that endometrial cells which are positive for the receptor are also positive for cytokeratin (epithelial cell marker). (G) Untransfected HEK-293 cells were used as negative control for immunostaining of CRH receptor protein; (H) the nuclei of the same cells stained with DAPI.

Figure 2 (A) RT-PCR analysis of CRH-R1 α and CRH-R1 β (panel I), CRH-R2 α (panel II), CRH-R2 α (panel III), CRH-R2 γ (panel IV) and β -actin (panel V) in human endometrial tissues. Lane M is the DNA ladder marker, lane 1 is cDNA from cell preparations, lane 2 is cDNA from endometrial tissue, lane 3 is the positive control and lane 4 is the negative control. (B) Displacement curves for binding of ^{125}I -oCRH and ^{125}I -UCN to human endometrial membranes. Each point is the mean of four estimations. (C) Autoradiograph of ^{125}I -oCRH cross-linked to its receptors in human endometrial membranes. The results are representative of three independent determinations.

Figure 3 (A) CRH-induced cAMP release from human endometrial membranes in the presence of different concentrations of h/rCRH. Human endometrial preparations (50 µg protein) were preincubated with different concentrations of h/rCRH (1–1000 nM), in 50 µl extraction buffer for 30 min at 22 °C, prior to the addition of 100 µl cAMP assay buffer at 37 °C. The reaction was terminated after 10 min and the amount of cAMP in the supernatants was determined by radioimmunoassay. Results are expressed as the mean±S.E.M. of four estimations from six independent experiments. **P*<0.05 compared with basal. (Insert) Identification of Gα-subunits photolabelled with [α -³²P]GTP-AA from human endometrial membranes in the presence of CRH. Membranes were incubated with CRH (100 nM), for 5 min at 30 °C before the addition of 5 µCi [α -³²P]GTP-AA for 3 min at 30 °C. Following centrifugation, membranes were placed on ice and exposed to ultraviolet light at a distance of 5 cm for 5 min. [α -³²P]GTP-AA-labelled G proteins were immunoprecipitated and were resolved on SDS-PAGE gels followed by autoradiography. Identical results were obtained from four independent experiments. (B) CRH-induced IP₃ accumulation from human endometrial membranes. Membranes (100 µg) were incubated with CRH (1–1000 nM) for 30 min at room temperature, followed by the addition of 200 µl IP₃ generation buffer and further incubation for 3 min at 37 °C. This was followed by extraction of inositol phosphates and neutralisation. IP₃ levels were determined by competitive binding assay. Results are expressed as the mean±S.E.M. of four estimations from three independent experiments. **P*<0.05 compared with basal. (Insert) Identification of Gq/11α-subunit photolabelled with [α -³²P]GTP-AA from human endometrial membranes in the presence of CRH (100 nM). Identical results were obtained from four independent experiments.

dose-dependent, showing a threshold of 10 nM CRH, while the maximal response ($210 \pm 15\%$ of basal) was observed at a concentration of 100 nM (Fig. 3A).

Activation of Gq/11 and the phospholipase C (PLC) system by CRH

Our studies have shown that CRH (100 nM) can also induce coupling to Gq/11 (4.7 ± 0.2 -fold increase above basal) ($P < 0.05$) (Fig. 3B insert). Activation of Gq/11 is predicted to stimulate PLC and increase generation of IP_3 . We found that CRH treatment of endometrial membranes induced a rapid IP_3 turnover, in a dose-dependent manner. This CRH effect has a threshold of 1 nM and a maximum response at 100 nM ($450 \pm 20\%$ of basal) (Fig. 3B).

Discussion

In this study we sought to examine the profile of CRH receptor expression and signalling properties in the human endometrium during the implantation phase. In our system we have used cultured epithelial endometrial cells, where CRH receptor was found to be present both at the mRNA and protein level. RT-PCR revealed the presence of at least three different CRH receptor subtypes: CRH-R1 α , CRH-R1 β and CRH-R2 α . Our data are in agreement with previously published work on endometrial CRH receptors (Di Blasio *et al.* 1997) and complement recent important findings regarding CRH and immunotolerance of the fetus (Makriganakis *et al.* 2001, 2003). Using ^{125}I -oCRH, we have confirmed the presence of specific, high-affinity binding sites in human endometrial membranes with an apparent molecular mass of 45 kDa.

The functional coupling of CRH receptors to Gs and Gq was further confirmed using second messenger studies where it was shown that CRH was able to induce both cAMP and IP_3 accumulation in a dose-dependent manner. Although the concentrations required for significant cAMP and IP_3 production were higher than the circulating levels of CRH, it is possible that the human endometrium is exposed to much higher concentrations of CRH due to the local production of the peptide (Makriganakis *et al.* 1995a).

Interestingly, using RT-PCR and specific primers for different receptor subtypes, we have found that CRH-R1 β was expressed only in 40% of tissues examined. The reasons for this inter-patient variation are unknown at present; however, steroid regulation of receptor splicing might be important. Our preliminary evidence suggests that that progesterone treatment affects CRH-R1 splicing at the mRNA level, upregulating CRH-R1 α and downregulating CRH-R1 β in human myometrial cells *in vitro* (Karteris *et al.* 2001a). It is attractive therefore to speculate that the down-regulation of CRH-R1 β might be due to the fact that, in this group of patients, all tissues examined had been exposed to increasing concentrations of progesterone.

Recent studies in our group have also shown that, despite the fact that CRH and UCN bind with the same affinity to the CRH-R1 α , they activate different intracellular signalling cascades (Grammatopoulos *et al.* 2000). For example, only UCN induces activation of the Erk1/2 pathway, and generates higher IP_3 responses when compared with CRH in cells expressing the CRH-R1 α receptor. A recent study has shown that Erk1/2 is activated during rat implantation and may play an important role during the decidualisation process (Thienel *et al.* 2002). It has also been shown that transient release of calcium from inositol 1,4,5-trisphosphate-specific stores regulates mouse preimplantation development (Stachecki & Armant 1996). It is therefore attractive to speculate that UCN can exert similar effects during human implantation.

We have also demonstrated expression of CRH-R2 α in human endometrium. It has recently been shown that UCN (one the native ligands for CRH-R2) is expressed in human endometrium (Florio *et al.* 2002). Therefore these data suggest the presence of distinct signalling networks involving UCN and CRH-R2 receptors. With the recent discovery of UCNII and UCNIII acting as native ligands for this receptor, it would be of interest to investigate whether these peptides are expressed in intrauterine tissues, and what signalling cascades they activate.

Our studies have shown that activation of CRH receptors led to stimulation of two signalling cascades: the Gs/AC and the Gq/ IP_3 . This is in accordance with previous studies from our laboratory that have shown that CRH receptors couple

to multiple G proteins in intrauterine tissues (Grammatopoulos *et al.* 1999b, Karteris *et al.* 2000). Our experiments have been carried out on material obtained from the secretory phase of the cycle, during which the endometrium prepares itself for implantation or, if implantation fails to take place, for menstruation. Thus, CRH acting via distinct signalling pathways might be implicated in the inflammatory phenomena that take place in the endometrium. For instance, activation of Gs can account for the induction of IL-6 (Zoumakis *et al.* 2000), FasL expression (Makrigiannakis *et al.* 2001) or for the generation of antiproliferative effects (Graziani *et al.* 2002), whereas coupling of CRH receptors to Gq could lead to induction of IL-1 (Zoumakis *et al.* 2000). During implantation, there is a major increase in vascular permeability surrounding the implanting embryo, with endometrial capillaries becoming dilated (Gravanis *et al.* 2001). CRH exerts a potent vasodilatory effect on the uterine artery (Vedernikov *et al.* 1999). Therefore, the presence of CRH receptors in epithelial cells and the coupling towards the Gq/IP₃ pathway could account for the regulation of endometrial vasculature, potentially via a nitric oxide-dependent pathway.

In conclusion, CRH via activation of specific CRH receptor and coupling to distinct G protein α -subunits may be involved in the sequence of events taking place in the endometrium before and during implantation in co-ordination with other factors deriving from the endometrium or the invading blastocyst. It is tempting to speculate that CRH, acting via different receptor subtypes, is able to exert different actions on the endometrium during the complex phenomena underlying its physiological changes. It is conceivable that abnormalities in this system might cause infertility. Therefore, an understanding of the physiological role of CRH could lead to novel therapy for some types of infertility or to novel forms of contraception.

Acknowledgements

Special thanks are due to the Consultants, Gynaecologists and Theatre Staff at the Women's Hospital, University Hospitals of Coventry and Warwickshire NHS Trust, Coventry, West Midlands, for supplying the endometrial biopsy

samples and to all patients for participating in this study. E K and N P should be considered first joint co-authors. This work was supported by grants from The Wellcome Trust and the General Charities of the City of Coventry.

References

- Aggelidou E, Hillhouse EW & Grammatopoulos DK 2002 Up-regulation of nitric oxide synthase and modulation of the guanylate cyclase activity by corticotropin-releasing hormone but not urocortin II or urocortin III in cultured human pregnant myometrial cells. *PNAS* **99** 3300–3305.
- Asakura H, Zwain HI & Yen SS 1997 Expression of genes encoding corticotropin-releasing factor (CRF) type 1 CRF receptor, and CRF-binding protein and localisation of the gene products in the human ovary. *Journal of Clinical Endocrinology and Metabolism* **82** 2720–2724.
- Athanassakis I, Farmakiotis V, Aifantis I, Gravanis A & Vassiliadis S 1999 Expression of the corticotropin-releasing hormone in the mouse uterus: participation in embryo implantation. *Journal of Endocrinology* **163** 221–227.
- Bernardini L, Moretti-Ro Jas I, Brush M, Ro Jas FJ & Balmaceda JP 1995 Status of hCG/LH receptor and G proteins in human endometrium during artificial cycles of hormone replacement therapy. *Journal of the Society for Gynecological Investigation* **2** 630–635.
- Di Blasio AM, Giraldo FP, Viganò P, Petraglia F, Vignali M & Cavagnini F 1997 Expression of CRH and its R1 receptor in human endometrial stromal cells. *Journal of Clinical Endocrinology and Metabolism* **82** 1594–1597.
- Fernandez-Shaw S, Shorter SC, Naish CE, Barlow DH & Starkey PM 1992 Isolation and purification of human endometrial stromal and glandular cells using immunomagnetic microspheres *Human Reproduction* **7** 156–161.
- Ferrari A, Petraglia F & Gursipide E 1995 Corticotropin-releasing factor decidualizes human endometrial stromal cells *in vitro*. Interaction with progesterin. *Journal of Steroid Biochemistry and Molecular Biology* **54** 251–255.
- Florio P, Arcuri F, Ciarmela P, Runci Y, Romagnoli R, Cintonio M, Di Blasio AM & Petraglia F 2002 Identification of urocortin mRNA and peptide in the human endometrium. *Journal of Endocrinology* **173** R9–R14.
- Grammatopoulos DK & Hillhouse EW 1999 Role of corticotropin-releasing hormone in onset of labour. *Lancet* **354** 1546–1549.
- Grammatopoulos D, Thompson S & Hillhouse EW 1995 The human myometrium expresses multiple isoforms of the corticotropin-releasing hormone receptor. *Journal of Clinical Endocrinology and Metabolism* **80** 2388–2393.
- Grammatopoulos DK, Dai Y, Randeava HS, Levine MA, Karteris E, Easton AJ & Hillhouse EW 1999 A novel spliced variant of the type 1 corticotropin-releasing hormone receptor with a deletion in the seventh transmembrane domain present in the human pregnant term myometrium and fetal membranes. *Molecular Endocrinology* **13** 2189–2202.
- Grammatopoulos DK, Randeava HS, Levine MA, Katsanou ES & Hillhouse EW 2000 Urocortin, but not corticotropin-releasing hormone (CRH), activates the mitogen-activated protein kinase signal transduction pathway in human pregnant myometrium: an effect mediated via R1 α and R2 β CRH receptor subtypes and stimulation of Gq-proteins. *Molecular Endocrinology* **14** 2076–2091.
- Gravanis A, Makrigiannakis A, Zoumakis E & Margioris AN 2001 Endometrial and myometrial corticotropin-releasing hormone (CRH): its regulation and possible roles. *Peptides* **22** 785–793.

- Graziani G, Tentori L, Portarena I, Barbarino M, Tringali G, Pozzoli G & Navarra P 2002 CRH inhibits cell growth of human endometrial adenocarcinoma cells via CRH-receptor 1-mediated activation of cAMP-PKA pathway. *Endocrinology* **143** 807–813.
- Hatzoglou A, Margioris AN, Bakogeorgou E, Gravanis A & Castanas E 1996 Identification, characterisation and localisation of corticotropin-releasing hormone receptors in human placenta. *Life Sciences* **59** 1871–1879.
- Hillhouse EW, Grammatopoulos D, Milton NG & Quartero HW 1993 The identification of a human myometrial CRH receptor which increases in affinity during pregnancy *Journal of Clinical Endocrinology and Metabolism* **76** 736–741.
- Karteris E, Grammatopoulos D, Dai Y, Olah KB, Ghobara TB, Easton A & Hillhouse EW 1998 Human placenta and fetal membranes express CRH 1 α and CRH C variant receptor. *Journal of Clinical Endocrinology and Metabolism* **83** 1376–1379.
- Karteris E, Grammatopoulos D, Randeva HS & Hillhouse EW 2000 Signal transduction characteristics of the corticotropin-releasing hormone (CRH) receptors in the feto-placental unit. *Journal of Clinical Endocrinology and Metabolism* **85** 1989–1996.
- Karteris E, Zervou S, Old RW, Grammatopoulos DG & Hillhouse EW 2001a Steroid-mediated expression of CRH-R1, vasopressin 1A and oxytocin receptors in the human myometrium. *Proceedings of the 83rd Endocrine Society Meeting*, Denver, CO, USA P3–623.
- Karteris E, Randeva HS, Grammatopoulos DK, Jaffe RB & Hillhouse EW 2001b Expression and coupling characteristics of the CRH and orexin type 2 receptors in human fetal adrenals. *Journal of Clinical Endocrinology and Metabolism* **86** 4512–4519.
- Linton EA, Woodman JR, Asboth G, Glynn BP, Plested CP & Bernal AL 2001 Corticotrophin releasing hormone: its potential for a role in human myometrium. *Experimental Physiology* **86** 273–281.
- Makrigiannakis A, Zoumakis E, Margioris AN, Theodoropoulos P, Stournaras C & Gravanis A 1995a The corticotropin-releasing hormone (CRH) in normal and tumoral epithelial cells of human endometrium. *Journal of Clinical Endocrinology and Metabolism* **80** 185–189.
- Makrigiannakis A, Margioris AN, Le Goascogne C, Zoumakis E, Nikas G, Stournaras C, Psychoyos A & Gravanis A 1995b Corticotropin-releasing hormone is expressed at the implantation sites of early pregnant rat uterus. *Life Sciences* **57** 1869–1875.
- Makrigiannakis A, Zoumakis E, Kalantaridou S, Coutifaris C, Margioris AN, Coukos G, Rice KC, Gravanis A & Chrousos GP 2001 Corticotropin-releasing hormone promotes blastocyst implantation and early maternal tolerance. *Nature Immunology* **2** 1018–1024.
- Makrigiannakis A, Zoumakis E, Kalantaridou S, Mitsiades N, Margioris A, Chrousos GP & Gravanis A 2003 Corticotropin-releasing hormone (CRH) and immunotolerance of the fetus. *Biochemical Pharmacology* **65** 917–921.
- Mastorakos G, Scopa CD, Vryonidou A, Friedman TC, Kattis D, Phenekos C, Merino MJ & Chrousos GP 1994 Presence of immunoreactive corticotropin-releasing hormone in normal and polycystic human ovaries. *Journal of Clinical Endocrinology and Metabolism* **79** 1191–1197.
- Nagata S 1994 Fas and Fas ligand: a death factor and its receptor. *Advances in Immunology* **57** 129–144.
- Noyers RW, Hertig AT & Rock J 1950 Dating the endometrial biopsy. *Fertility and Sterility* **1** 3–25.
- Offermanns S, Schultz G & Rosenthal W 1991 Identification of receptor-activated G proteins with photoreactive GTP analog, [α -³²P]GTP azidoanilide. *Methods in Enzymology* **195** 286–301.
- Petraglia F, Sawchenko PE, Rivier J & Vale W 1987 Evidence for local stimulation of ACTH secretion by corticotropin-releasing factor in human placenta. *Nature* **328** 717–719.
- Petraglia F, Tabanelli S, Galassi MC, Garuti GC, Mancini AC, Genazzani AR & Gursipide E 1992 Human decidua and *in vitro* decidualised endometrial stromal cells at term contain immunoreactive corticotropin-releasing factor (CRF) and CRF messenger ribonucleic acid. *Journal of Clinical Endocrinology and Metabolism* **74** 1427–1431.
- Riley SC, Walton JC, Herlick JM & Challis JR 1991 The localisation and distribution of CRH in the human placenta and fetal membranes throughout gestation. *Journal of Clinical Endocrinology and Metabolism* **72** 1001–1007.
- Selam B, Kayisli UA, Mulayim N & Arici A 2001 Regulation of Fas ligand expression by estradiol and progesterone in human endometrium. *Biology of Reproduction* **65** 979–985.
- Stachecki JJ & Armant DR 1996 Transient release of calcium from inositol 1,4,5-trisphosphate-specific stores regulates mouse preimplantation development. *Development* **122** 2485–2496.
- Thienel T, Chwalisz K & Winterhager E 2002 Expression of MAPkinases (Erk1/2) during decidualization in the rat: regulation by progesterone and nitric oxide. *Molecular Human Reproduction* **8** 465–474.
- Vedernikov YP, Saade GR, Chwalisz K & Garfield RE 1999 Endothelium-dependent and -independent mechanisms of vasorelaxation by corticotropin-releasing factor in pregnant rat uterine artery. *Journal of Pharmacology and Experimental Therapeutics* **288** 407–413.
- Zoumakis E, Margioris AN, Stournaras C, Dermitzaki E, Angelakis E, Makrigiannakis A, Koumantakis E & Gravanis A 2000 Corticotrophin-releasing hormone (CRH) interacts with inflammatory prostaglandins and interleukins and affects the decidualization of human endometrial stroma. *Molecular Human Reproduction* **6** 344–351.

Received in final form 26 September 2003

Accepted 29 September 2003