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

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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 1α (CRH-R1α) and CRH-R2α in the human endometrium. CRH-R1β was present in 40% of endometrial cDNAs examined. No apparent expression of CRH-R2β, CRH-R2γ 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.

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 (Makrigiannakis et al. 1995α). 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. 2001).

In rodents, immunoneutralisation of CRH or blockage of the CRH-R1 receptor site with the antagonist antalarmin decreases the number of live embryos (Athanassakis 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 flasks (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 CTAGCTGCCCCGCAGGGCTGCAGGGCCCA GCAGGTGCC). Hybridisation solution (100 µl) containing 1 ng/µl of the probe, was allowed to hybridise at 37 °C overnight. Slides were then
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 RNeasy™ 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. 2001). 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 1 List of primers used for the amplification of CRH receptor subtypes in human endometrium

<table>
<thead>
<tr>
<th>Primer</th>
<th>Subtype</th>
<th>Sense</th>
<th>Antisense</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRH-R1α/β</td>
<td>Sense</td>
<td>5′-GGCAGCTAGTGGTTCCGGCC-3′</td>
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<tr>
<td>CRH-R1c</td>
<td>Sense</td>
<td>5′-TGCGAGCCACCCGGATGCTC-3′</td>
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<tr>
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<tr>
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<td>Sense</td>
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<tr>
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<td>Sense</td>
<td>5′-CCCTCAACCAACCTCGAGTCC-3′</td>
<td></td>
</tr>
<tr>
<td>CRH-R2γ</td>
<td>Sense</td>
<td>5′-CTCAAGGATCCTGTGCAT-3′</td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>Sense</td>
<td>5′-GGCTCACACTGTGAGTAGTT-3′</td>
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</tbody>
</table>

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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 µl125I-ovine (o) CRH and125I-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 carefully removed, 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 with125I-oCRH for 2 h in 300 µl buffer (50 mM Tris-Cl, 2 mM EGTA, 10 mM MgCl₂ and 1.5 g/1 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 23 °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

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

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Size (kDa)</th>
<th>α-subunit</th>
<th>Sequence</th>
<th>Binding site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gs</td>
<td>47.45</td>
<td>α s</td>
<td>RMHLROQYELL</td>
<td>C-terminus</td>
</tr>
<tr>
<td>Go</td>
<td>40.5</td>
<td>α o</td>
<td>GCTLSAEERAALERSK</td>
<td>N-terminus</td>
</tr>
<tr>
<td>Gi</td>
<td>41</td>
<td>α i₁/₂</td>
<td>KENLKDCLGF</td>
<td>C-terminus</td>
</tr>
<tr>
<td>Gq</td>
<td>42</td>
<td>α α₁/₁₁</td>
<td>QLNKKEYNLV</td>
<td>C-terminus</td>
</tr>
</tbody>
</table>
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.

**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

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**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 ³⁵H[IP₃] from a specific bovine adrenocortical IP₃ binding protein. The interassay coefficient of variation was 11·7%.
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-R1α or CRH-R1β receptor subtypes we were able to detect CRH-R1α in endometrial biopsy samples and epithelial cells. However, only 40% of the cDNAs encoded CRH-R1β 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-R2α in the human endometrium (Fig. 2A). None of the other type-2 receptor subtypes (CRH-R2β and CRH-R2γ) 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 125I-oCRH and 125I-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 125I-oCRH with the unrelated peptide AVP (at concentrations up to 100 nM) which was unable to displace 125I-oCRH and 125I-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 Gsα and adenylate cyclase system by CRH**

In order to identify the G proteins activated by CRH, we use the non-hydrolysable GTP analogue [α-32P]GTP-γS which binds – upon receptor stimulation – to the GTP-binding site of activated Ga proteins. Immunoprecipitation with specific Ga 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 Gsα subunits (47 and 45 kDa) were activated by CRH receptors. CRH increased the incorporation of GTP-γS 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.

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Figure 2  (A) RT-PCR analysis of CRH-R1α and CRH-R1β (panel I), CRH-R2α (panel II), CRH-R2α (panel III), CRH-R2γ (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 Gsα-subunits photolabelled with \[^{\gamma}\text{S}^{32}\text{P}]\text{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 \[^{\gamma}\text{S}^{32}\text{P}]\text{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. \[^{\gamma}\text{S}^{32}\text{P}]\text{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 \[^{\gamma}\text{S}^{32}\text{P}]\text{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 ± 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 ± 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$\alpha$, CRH-R1$\beta$ and CRH-R2$\alpha$. 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 (Makrigiannakis et al. 2001a, 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 (Makrigiannakis et al. 1995a).

Interestingly, using RT-PCR and specific primers for different receptor subtypes, we have found that CRH-R1$\beta$ 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$\alpha$ and downregulating CRH-R1$\beta$ in human myometrial cells in vitro (Karteris et al. 2001a). It is attractive therefore to speculate that the down-regulation of CRH-R1$\beta$ 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$\alpha$, 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$\alpha$ 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$\alpha$ 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

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*E KARTERIS and others · CRH receptors in human endometrium*
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.

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References


