Identification of a Novel Corticotropin-Releasing Hormone Type 1β-Like Receptor Variant Lacking Exon 13 in Human Pregnant Myometrium Regulated by Estradiol-17β and Progesterone

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Two types of CRH receptors mediate the diverse biological functions of CRH and CRH-related peptides. The type 1 CRH-R (CRH-R1) is extensively targeted by pre-mRNA splicing mechanisms that give rise to multiple mRNA splice variants. RT-PCR amplification of CRH-R1 sequences from human myometrium yielded cDNAs that encode a novel CRH-R1 splice variant with structural characteristics identical with CRH-R1β except a 14-amino acid deletion in the seventh transmembrane domain characteristic of the CRH-R1d. Transient expression of the hybrid CRH-R1 variant (CRH-R1β/d) in human embryonic kidney 293 cells revealed primarily intracellular expression, although some plasma membrane protein expression was also detectable. CRH bound to CRH-R1β/d with affinity comparable with the CRH-R1β; however, it was unable to stimulate adenylyl cyclase or other second messengers. Using a semiquantitative RT-PCR assay, CRH-R1β/d mRNA transcript was detected in human pregnant, but not nonpregnant, myometrium as early as 31 wk of gestation. Furthermore, in human pregnant myometrial cells, the relative expression of CRH-R1β and CRH-R1β/d mRNA appeared to be regulated by steroids; CRH-R1β/d mRNA expression was increased by estradiol-17β, whereas CRH-R1β mRNA levels were increased by progesterone. Progesterone also substantially increased CRH-R1α mRNA levels and cellular responsiveness to CRH as determined by increased agonist binding and cAMP production as well as resistance to CRH-R heterologous desensitization by phorbol esters. These results provide novel evidence for distinct patterns of CRH-R1 splicing and identify specific steroid-mediated regulation of CRH-R1 variant expression, which might be important for modulating CRH actions during human pregnancy and labour. **(*Endocrinology* 151: 4959–4968, 2010)**

During human pregnancy, the placenta synthesizes and secretes CRH into the maternal circulation. This placental CRH might act to control a placental clock, which is active from the early stages of pregnancy and determines the length of the gestation and the timing of parturition and delivery (1). The biological roles of CRH during pregnancy are still not fully understood; the presence of specific CRH receptors (CRH-Rs) in many tissues of the fetomaternal unit such as the placenta, myometrium, decidua, and fetal adrenals, suggests that CRH might target these tissues to exert important functions (2). For example, the expression of functional CRH-R and locally produced CRH and CRH-related peptides such as urocortin (Ucn)-1 and Ucn2 in the myometrium (3–6), which regulate smooth muscle contractility, suggests a role in the development of human parturition by preventing the premature

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Abbreviations: Bmax, Maximum binding site concentration; CRH-R, CRH receptor; E_2, estradiol-17β; FCS, fetal calf serum; GPCR, G protein-coupled receptor; HEK, human embryonic kidney; IQ, ImageQuant; Kd, dissociation constant; o, ovine; P4, progesterone; 4α-PDD, 4α-phorbol-12,13-didecanoate; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; TMD, transmembrane domain; Ucn, urocortin.

activation of the myometrium and thus coordinating the transition of the uterus from a state of relaxation to one of contraction (7).

The actions of CRH in target tissues are mediated via binding to and activation of specific seven-transmembrane domain (TMD) G protein-coupled receptors (GPCRs). To date, two distinct CRH receptor subtypes (R1 and R2) have been identified in mammals (8, 9). These receptors belong to the *Secretin* family of GPCRs (receptors for brain-gut neuropeptides) and exhibit a diverse alternative pre-mRNA splicing pattern that appears to be conserved among members of the *Secretin* family of GPCRs (10). The fully functional CRH-R1, termed CRH-R1α, is a 415-amino acid protein that binds both CRH and Ucn1 and activates multiple G proteins and downstream effectors in a tissue-dependent manner (11, 12). In the human myometrium, as well as in other tissues, multiple CRH-R1 mRNA splice variants have been identified, including the CRH-R1β variant, which contains 29 additional amino acids in the first intracellular loop (11), and shows reduced binding affinity for CRH and coupling to downstream effectors. Another is the CRH-R1d variant (13) with a 14-amino acid deletion from the seventh TMD, which does not affect binding affinity but severely impairs signal transduction characteristics. Although the physiological roles of these receptor variants is poorly understood, recent evidence suggests that they might dampen CRH cellular responsiveness by acting as decoy receptors capable of competing with the fully active receptors for agonist binding and absorbing peptide bioactivity and also as potential dominant-negative regulators when coexpressed with the fully active receptors to inhibit downstream signaling activity (14). There is also evidence to suggest that receptor variants like the CRH-R1β exhibit unique responses to posttranslational regulatory mechanisms such as Ser/Thr kinase-induced receptor phosphorylation (15).

During pregnancy, the expression of the CRH-R1 variants appears to be dynamically regulated by unknown mechanisms, and the onset of labor (preterm or term) is associated with increased transcription of the myometrial *CRH-R1* gene and altered splicing events that down-regulate the mRNA encoding the CRH-R1β but up-regulate receptor variants such as CRH-R1d (16, 17). In this study, we report the cloning and characterization of a novel cDNA from human pregnant but not nonpregnant myometrial RNA that encodes a novel spliced variant of the human CRH-R1. This CRH-R1 variant contains an elongated first intracellular loop by 29 amino acids similar to the CRH-R1β together with a deletion of 14 amino acids from the putative seventh TMD. This hybrid CRH-R1 variant was termed CRH-R1β/d. We also characterized the binding and functional properties of this CRH-R1 variant by overexpression studies in human embryonic kidney (HEK)-293 cells, and we investigated potential mechanisms regulating CRH-R1 mRNA variant expression in human pregnant myometrial cells.

Materials and Methods

Chemicals

Radioiodinated ovine (o) CRH, human/rat CRH, and Ucn1 were obtained from Bachem (United Kingdom) Ltd. (Merseyside, UK). Mouse monoclonal vimentin antibody and anti-mouse IgG-tetramethylrhodamine isothiocyanate conjugated were obtained from Sigma Aldrich United Kingdom (Poole, UK). Mouse monoclonal muscle-actin antibody was obtained from Dako Ltd. (Crawley, UK). The mammalian expression vector pcDNA3.1(−) and Lipofectamine were obtained from Invitrogen (Paisley, UK). CRH-R1 antibody (polyclonal antibody raised against a peptide mapping at the C terminus of human CRH-R1) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Phospho-ERK1/2 (Thr202/Tyr204), total ERK2, phospho-(Thr180/Tyr182), and total p38 MAPK antibodies were from Cell Signaling (Chandlers Ford, Hampshire, UK). Deoxynucleotide triphosphates and the DNA ladder were purchased from Fermentas Life Sciences (York, UK). Synthetic oligonucleotide probes and enzymes were purchased from Life Technologies (Paisley, UK). GeneElute mammalian total RNA kit and all other chemicals were purchased from Sigma Chemical Co. Ltd. (Poole, Dorset, UK).

Experimental subjects and sample preparations

Pregnant myometrial biopsies (n = 13) were obtained from women undergoing elective cesarean section at term (n = 7) or preterm (n = 6) before the onset of labor for nonmaternal problems. The biopsy site was standardized to the upper margin of the lower segment of the uterus in the midline. This provides the closest approximation to the upper segment of the uterus. Nonpregnant myometrial tissues (n = 6) were obtained from premenopausal controls undergoing hysterectomy for nonmalignant conditions. The nonpregnant myometrial biopsies were obtained from the same location as the cesarean section myometrial biopsies to avoid possible differences in receptor expression patterns. The relative content of myometrial and fibrous tissue in these biopsies was identified by immunostaining using specific smooth muscle cell and fibroblast markers (actin and vimentin, respectively). The biopsies were immediately snap frozen in liquid nitrogen and subsequently stored at −70 C until use or were processed for myocyte cell culture. Ethical approval was obtained from the local ethical committee and informed consent to the study was obtained from all patients.

Preparation of myometrial cell cultures

Myocytes were prepared by enzymatic dispersion as previously described (17). Briefly, pieces of myometrium were transferred into DMEM containing collagenase (300 U/ml), deoxyribonuclease (30 U/ml), penicillin (200 U/ml), and streptomycin (200 mg/ml) and incubated at 37 C for 30 min. After filtration and centrifugation, cells were suspended in DMEM containing 10% fetal calf serum (FCS), penicillin (100 U/ml), streptomycin

(100 mg/ml), and Fungizone (2.5 μg/ml). The cells were kept at 37 C in a humidified atmosphere of 95% air-5% CO_2 until confluent (~2 wk). The purity of myometrial muscle cells was assessed by immunocytochemical staining. Mouse antihuman smooth muscle actin-specific monoclonal antibody and peroxidase-conjugated rabbit antimouse antibody were used. The human fibroblast cells and omission of the primary antibody were used as negative controls, whereas frozen myometrial tissue was used as a positive control. To minimize fibroblast contamination, the myocyte preparation was repurified 48 h before the experiments using 0.5% trypsin.

To study the effects of steroid hormones on myometrial CRH-R1-spliced variant expression, charcoal-dextran T-70-treated FCS (Life Technologies) was used, and the pH indicator, phenol red, was omitted. The hormone-depleted FCS was used in the same proportions as those described previously. For time-course experiments, myometrial cells were treated with 0.5–5 μM of estradiol-17β (E_2) or progesterone (P4) or both for various time intervals (0–24 h). At the end of the incubation period, cells were collected, pelleted by centrifugation, and stored at −80 C until RNA extraction.

RNA extraction and semiquantitative RT-PCR

Total RNA was extracted from pregnant and nonpregnant myometrium tissues or myometrial cell cultures and reverse transcribed to synthesize cDNA by using RNase H reverse transcriptase (Life Technologies). Distilled water was used in place of the cDNA as a negative control for each reaction. The forward primer (position, bp 434–452; sequence, 5′-GGCCAGGCTGCACCCATTG-3′) was designed to hybridize with a nucleotide sequence present within exon 6 (which encodes for the 29 amino acid insert in the first intracellular loop, and it is therefore specific for the CRH-R1β), whereas the reverse primer (position, bp 1315–1334; sequence, 5′-TTCGTCAGGTGTCGTCAGAC-3′) designed to hybridize with a nucleotide sequence present within exon 14 (common to all the CRH-R1 subtypes). The semiquantitative RT-PCR method was carried out as previously described (18). The linearity and reproducibility of the PCR were optimized over a wide range of cycle numbers (n = 20–45) with different amounts of cDNA. In addition, different amounts of *in vitro*-transcribed RNA corresponding to the different CRH-R1 cDNA variants cloned in the pBluescript vector (Stratagene, La Jolla, CA) were used to test potential preferential amplification of one receptor variant over the other. The PCR was performed in 25-μl reactions using 10 μCi/μl [^{32}P]-labeled α-dGTP and 5 IU/μl TAQ DNA polymerase (Life Technologies). Amplifications were carried out as follows: an initial denaturation step of 5 min at 94 C, followed by 35 cycles for 35 cycles of 30 sec at 94 C, 1 min at 58 C, and 1 min at 72 C, followed by a final extension at 72 C for 10 min. PCR products were separated by electrophoresis on a 6% polyacrylamide gel and the dried gel exposed to x-ray film (Fuji Photo Film Co. Ltd., Tokyo, Japan) or to a PhosphorImager screen (Molecular Dynamics, Pharmacia Amersham Life Sciences, Little Chalfont, UK) for quantification purposes. Radioactivity peaks for each product were determined using ImageQuant (IQ; Molecular Dynamics, Pharmacia Amersham Life Sciences).

Real-time RT-PCR

Real-time, one-step PCR for CRH-R1α and CRH-R1β mRNA (271 and 107 bp, respectively) was performed using the LightCycler thermal cycler system (Roche Diagnostics, Burgess Hill, West Sussex, UK) as previously described (19). The primers used for the CRH-R1 were: CRH-R1α, 5′-GGCAGCTAGTGGTTCGGCG-3′ (sense) and 5′-TCGCAGGCACCGGATGCTC-3′ (antisense); CRH-R1β, 5′-GGCCAGGCTGCACCC ATTG-3′ (sense), and 5′-TCGCAGGCACCGGATGCTC-3′ (antisense). The quantification data were analyzed with the LightCycler analysis software (Roche Diagnostics).

Cloning and sequence analysis

For sequencing purposes, DNA fragments were purified from a 1.6% agarose gel by using QIAquick gel extraction kit (QIAGEN, Crawley, West Sussex, UK) and sequenced using internal primers for the whole gene in an automated DNA sequencer, and the sequence data were analyzed using Blast Nucleic Acid Database Searches from the National Center for Biotechnology Information (Bethesda, MD).

The full-length CRH-R1 variant cDNAs were generated by RT-PCR of human pregnant myometrial cell poly RNA and were subsequently inserted into the plasmid pBluescript II SK (+/−)-derived T vector and transformed into *Escherichia coli* strain XL-1 Blue, and the positively selected clones were subcultured, as previously described (13).

Transfection of HEK-293 cells

The cDNAs encoding CRH-R1 variants were subcloned in pcDNA 3.1(−) (Invitrogen) HEK-293 cells, grown in DMEM, and transiently transfected using Lipofectamine reagent (Life Technologies) according to the manufacturer's instructions. Transfections were performed in 60-mm dishes (70% confluent cells) with 8 μg CRH-R1 plasmid cDNA and 16 μg Lipofectamine reagent. Receptor expression was allowed to proceed for 24 h.

Western blot analysis and confocal microscopy of CRH-R1 variant expression

Confluent HEK-293 transient expressing CRH-R1 variants were washed with PBS and lysed with 0.2% NaCl. The cells were homogenized in extraction buffer A [10 mM Tris-HCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 mM $MgCl_2$, 0.1% BSA, and 15 mM bacitracin (pH 7.2)] in Dulbecco's PBS. The homogenate was centrifuged at 600 × *g* for 30 min at 4 C to remove nuclei and unbroken cells. The supernatant was collected and centrifuged at 40,000 × *g* for 60 min at 4 C. The pellet (50 μg in 50 μl aliquots) was rinsed twice and were solubilized in 5 M urea-containing Laemmli buffer (0.17 M sodium dodecyl sulfate; 0.4 M dithiothreitol; and 50 mM Tris-HCl, pH 8.0), mixed, and placed in a boiling water bath for 5 min and allowed to cool at room temperature.

Western blot analysis and immunofluorescent confocal microscopy in HEK-293 and human myometrial smooth muscle cells using primary antibody for the CRH-R1/2 (Santa Cruz Biotechnology) and a Leica model DMRE laser-scanning confocal microscope (Leica Microsystems, Buckinghamshire, Milton, Keynes, UK) with TCS SP2 scan head were carried out as previously described (14, 17).

Binding and second-messenger assays

Binding characteristics of ^{125}I-oCRH using Scatchard analysis and cAMP, inositol phosphate, ERK1/2, and p38 MAPK

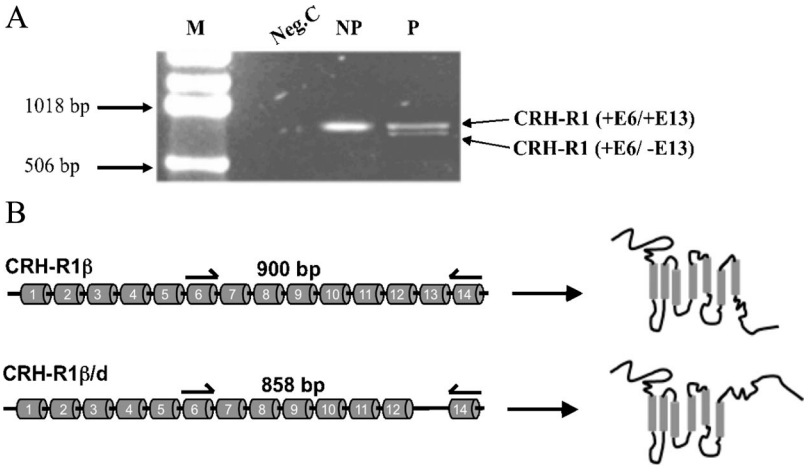

FIG. 1. Identification of exon 6-positive CRH-R1 mRNA variants in human myometrium. A, RT-PCR amplification of CRH-R1 variants sequences from RNA extracted from nonpregnant (NP) or term pregnant (P) human myometrial biopsies. Specific primers able to amplify exons 6–14 within CRH-R1β mRNA were used as described in *Materials and Methods*. PCR products were resolved on 1.6% agarose gel and stained with ethidium bromide. The identities of the fragments were confirmed by direct nucleotide sequencing. The cDNA sequence has been submitted to the GenBank database (identification no. 1367279). B, Schematic diagram of CRH-R1β and -R1β/d exonic sequence modifications and the predicted protein structure. *Arrows* indicate the annealing position of PCR primers.

stimulation assays of HEK-293 cells transfected with the different CRH-R1 variants were carried out as previously described (13, 14) in cells plated into 24-well plates at a density of 10^5 cells/well. cAMP production was measured using a cAMP RIA kit (Biomedical Technologies Inc., Stoughton, MA).

Statistical analysis

Data are shown as the means ± SEM of each measurement. Data were tested for homogeneity and comparison between group means was performed by one- or two-way ANOVA. $P < 0.05$ was considered significant.

Results

Identification of a novel CRH-R1 variant mRNA in human myometrium

Using specific RT-PCR primers to amplify CRH-R1 exon 6-containing cDNA sequences, two DNA fragments of 900 and 858 bp, respectively, were amplified from pregnant but not nonpregnant myometrial biopsies (Fig. 1A). After nucleotide sequencing, the nucleic acid sequence of the 900 fragment was shown to be identical with the CRH-R1β, whereas the nucleic acid sequence of the 858 fragment was shown to be identical with the CRH-R1β except for an in-frame 42-bp deletion that corresponds to exon 13 of the human CRH-R1 and encodes 14 amino acids (Gly^{385}-Glu^{398}) on the putative seventh TMD of the receptor protein, suggesting that this novel CRH-R1 splice variant (termed CRH-R1β/d) is generated by alternative splicing of exon 13 but retention of exon 6 (characteristic of the CRH-R1β sequence) (Fig. 1B). Hydropathy analysis of the amino acid sequences of the CRH-R1β and CRH-R1β/d receptors demonstrated that the hydrophobic region corresponding to the seventh TMD was smaller, consistent with the predicted 14-amino acid deletion (data not shown).

The length of the amplified nucleotide sequence required to specifically detect mRNA transcripts with exon 6 inclusion and exon 13 excision prohibited the use of real-time quantitative RT-PCR. Therefore, CRH-R1β/d mRNA expression in term or preterm myometrium (31–40 wk of gestation) was investigated by a semiquantitative RT-PCR assay. Results showed that CRH-R1β/d mRNA expression was highest in early third-trimester myometrium (wk of gestation) and progression toward term significantly decreased expression (Fig. 2); in contrast CRH-R1β mRNA expression levels in nonlaboring myometrium remained constant between 31 and 35 wk of gestation, whereas at term CRH-R1β mRNA expression was significantly elevated, in agreement with our previous data (17).

Cell expression, binding, and signal transduction characteristics of the CRH-R1β/d receptor

Expression of the variant CRH-R1β/d receptor was determined in a transient transfection HEK-293 cellular system by using immunoblotting analysis to determine the apparent molecular weight of the CRH-R in plasma membranes and compare protein expression in HEK-293 cells transiently expressing CRH-R1β (293-R1β) and CRH-R1d (293-R1d) receptors. Fractionation of total cell lysates by SDS-PAGE was performed and immunoblotting using an antibody raised against a peptide corresponding to amino acids 425–444 in the C terminus of the human CRH-R1/2 precursor. Western blot analysis using the CRH-R1/2 antibody in 293-R1β, 293-R1d, or 293-R1β/d cell lysate preparations showed a single immunoreactive protein with an apparent molecular mass of approximately 50 kDa (Fig. 3A). Using densitometric analysis, it was shown that there was no significant difference in the expression levels of all CRH-R variants tested, thus confirming that transfection efficiencies were not different for all CRH-R1 variants. No immunoreactive proteins were detected in untransfected HEK-293 cells or when 1 μM of a synthetic CRH-R1 blocking peptide was added, thus confirming the specificity of these data (data not shown). Indirect fluorescent confocal microscopy using a specific CRH-R1/2 antibody was also performed to determine CRH-R1β/d sub-

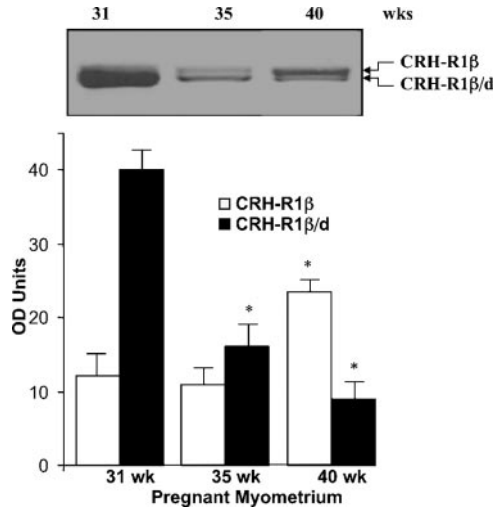

FIG. 2. Determination of CRH-R1β and -R1β/d mRNA expression levels in human myometrium during the third trimester of pregnancy. Representative autoradiograph (*top panel*) of CRH-R1β and -R1β/d mRNA sequence amplification by a semiquantitative RT-PCR as described in *Materials and Methods.* PCR products were separated by gel electrophoresis, and the dried gel was exposed to x-ray film. The intensity of bands was quantified by the Microsoft Windows IQ program after scanning of the screen (*bottom panel*). Data represent the mean ± SEM of three estimations from three independent experiments. *, $P < 0.05$ compared with values obtained at 31 wk.

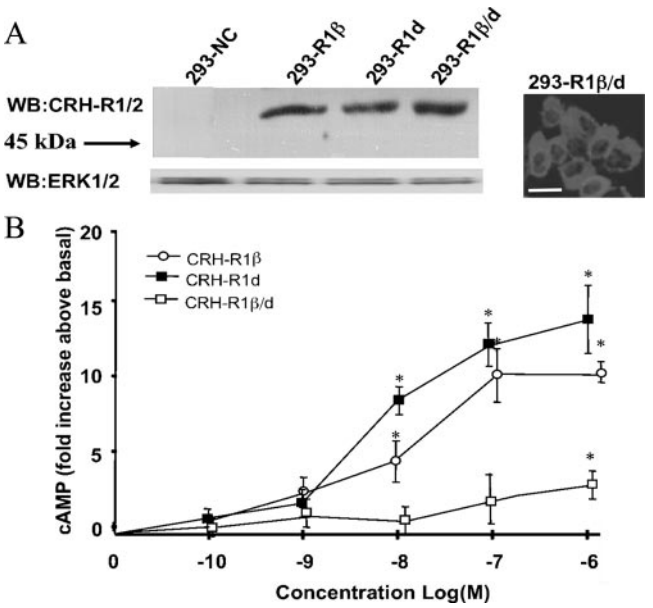

FIG. 3. Expression and signaling characteristics of CRH-R1β/d expressed in HEK-293 cells. A, Cell lysates from HEK-293 cells (untransfected-NC) or transiently expressing CRH-R1β or CRH-R1d or CRH-R1β/d were prepared, and 50 μg of protein was fractionated on SDS-PAGE and subjected to immunoblotting with specific antibodies against CRH-R1/2 or total ERK1/2. Antibody complexes were detected by enhanced chemiluminescence. *Inset*, CRH-R1β/d subcellular localization was assessed by indirect confocal microscopy. HEK-293 cells transiently expressing CRH-R1β/d were grown on poly-D-lysine-coated glass cover slips. Receptor immunoreactivity was detected with a RH-R1/2-specific antibody and Alexa Fluor 594 secondary antibody. Identical results were obtained from three independent experiments. WB, Western blot. *Scale bar*, 30 μm. B, cAMP release from HEK-293 cells transiently expressing CRH-R1β/d or its parent receptor variants CRH-R1β or CRH-R1d in the presence of different concentrations of CRH. Results are representative of one receptor clone and are expressed as the mean six SEM of four estimations from three independent experiments. *, $P = 0.05$ compared with basal.

cellular localization. Results (Fig. 3A, *inset*) showed significant intracellular accumulation of immunofluorescent signal in CRH-R1β/d-overexpressing cells with a weak plasma membrane signal similar to R1d and in contrast to CRH-R1β-overexpressing cells that showed immunostaining that was exclusively localized around the plasma membrane in agreement with our previous data (15).

The binding characteristics of the novel CRH-R1 variant were determined using a specific radioreceptor assay in membranes prepared from 293-R1β/d cells and compared with those of the 293-R1β and 293-R1d cells. Scatchard analysis of ^{125}I-oCRH binding for each of the CRH-R1 variants was consistent with the presence of a single population of high-affinity receptors; the CRH-R1β/d and CRH-R1β displayed identical binding affinity for CRH with apparent dissociation constants (Kd) of 18.90 ± 1.4 and 20.60 ± 1.5 nM for CRH-R1β/d and CRH-R1β, respectively. In contrast, the CRH-R1d exhibited significantly higher binding affinity for CRH (2.2 ± 0.7 nM). The maximum binding site concentrations (Bmax) in the plasma membrane were significantly lower for the R1d and R1β/d variants compared with R1β (0.32 ± 0.9 and 0.6 ± 1.5 *vs.* 5.1 ± 0.6 nmol/mg protein).

The signal transduction characteristics of the R1β/d receptors were also characterized and compared with those of R1β and R1d. In both 293-R1β and 293-R1d cells, CRH elicited a dose-dependent modest increase in cAMP production with a threshold of 10 nM and maximum effect observed at CRH concentrations of 100 nM. The maximum cAMP response ranged between 11 ± 2- and 16 ± 3-fold above basal (Fig. 3B). However, in 293-R1β/d cells, the effect of CRH on adenylyl activation was minimal and CRH was able to stimulate a weak cAMP response only at concentrations greater than 1 μM (3 ± 1.3-fold above basal). In all types of cells, the integrity of adenylyl cyclase was tested by the use of forskolin (10^{-5} M), and no differences were found (data not shown). Furthermore, HEK-293 cells expressing R1β, R1d, or R1β/d variant receptors showed no increase in inositol 1,4,5-triphosphate production or ERK1/2 and p38 MAPK phosphorylation to CRH challenge (with concentrations up to 1 μM) (data not shown). Similar results were obtained when Ucn1 was used as the stimulating agonist (data not shown).

Steroid regulation of CRH-R1 variant mRNA expression

Progression toward term is associated with alterations in the E_2-P4 balance that favor an increasingly estrogenic

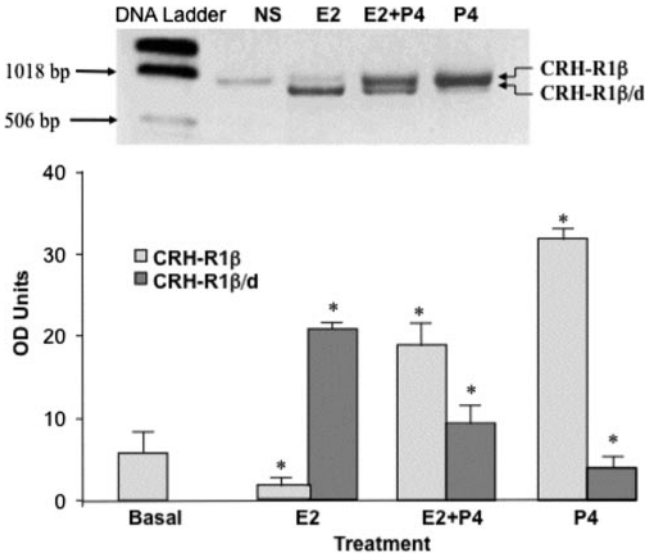

FIG. 4. Effect of E_2 and progesterone on CRH-R1β and CRH-R1β/d mRNA expression levels in human myometrial smooth muscle cells. RNA was extracted from cells pretreated with 5 μM of E_2 or P4 or both for 12 h, and CRH-R1β and R1β/d mRNA sequence were amplified by a semiquantitative RT-PCR as described in *Materials and Methods.* PCR products were separated by gel electrophoresis and the dried gel was exposed to x-ray film. A representative autoradiograph is shown in the *top panel.* The intensity of bands was quantified by the Microsoft Windows IQ program after scanning of the screen (*bottom panel*). Data represent the mean ± SEM of three estimations from three independent experiments. *, $P < 0.05$ compared with control [untreated (NS, Nonstimulated)] cells.

environment (20). To investigate potential effects of steroid hormones on the mRNA expression of R1β and R1β/d variants, human myometrial smooth muscle cells isolated from pregnant term nonlaboring myometrium and cultured in hormone depleted serum were treated with or without 5 μM of E_2 or P4 or both. Under basal conditions only the CRH-R1β mRNA was detected (Fig. 4). Treatment of cells with E_2 for 12 h resulted in a 4-fold decrease in the amount of CRH-R1β mRNA, and this was associated by a substantial up-regulation of exon 13-negative CRH-R1β/d mRNA transcripts. In E_2-treated myometrial cells, the CRH-R1β/d appeared to be the predominant exon 6-containing CRH-R1 variant mRNA. The effects of E_2 on CRH-R1β and R1β/d mRNA transcripts was found to be time dependent and were apparent 6 h after the start of the hormonal treatments and were sustained for at least 24 h (data not shown). Similar effects were observed when E_2 was used at a concentration range of 0.5–5 μM; however, 5 μM E_2 produced the most consistent responses and was used in all subsequent experiments (data not shown).

Treatment of myometrial cells with E_2 in the presence of P4 markedly decreased E_2 effects on CRH-R1β/d mRNA expression but increased CRH-R1β mRNA transcripts. In these cells the CRH-R1β appeared to be the predominant exon 6-containing CRH-R1 variant mRNA. Treatment of myometrial cells with P4 alone substantially increased CRH-R1β mRNA transcripts (Fig. 4A), thus confirming P4 actions on stimulating CRH-R1β mRNA expression. Interestingly, an apparent up-regulation in both exon 13-positive (CRH-R1β) and -negative (CRH-R1β/d) R1 variant mRNAs was evident in P4-treated cells, raising the possibility of stimulatory effects at the level of the *CRHR1* promoter.

To investigate whether P4 specifically targeted exon 6 positive (CRH-R1β and CRH-R1β/d) mRNA transcripts or was able to influence expression of other CRH-R1 mRNA transcripts, real-time RT-PCR was used to analyze expression of exon 6-negative CRH-R1 mRNA transcripts, such as the CRH-R1α mRNA. Indeed, we identified a significant increase by 3-fold of exon 6-negative CRH-R1 mRNA transcripts after P4 treatment (Fig. 5A). We also used indirect confocal immunofluorescent microscopy studies to investigate potential changes in CRH-R subcellular localization in P4-treated cells (Fig. 5B). These studies failed to detect any significant changes in distribution of CRH-R immunofluorescent signal in response to P4 treatment, although the overall CRH-R immunoreactivity appeared enhanced in P4-treated cells.

Given that the principal exon 6-negative CRH-R1 mRNA transcript encodes for the fully active CRH-R1α, we also investigated the possibility that P4 pretreatment of myometrial cells alters cellular responsiveness to the actions of CRH and stimulation of downstream signaling events. After P4 pretreatment, the ability of the myometrial cells to increase intracellular cAMP levels in response to CRH (1–100 nM) was significantly enhanced by 2- to 4-fold (Fig. 6A). There was no differences in forskolin-stimulated cAMP levels between control and P4-treated cells, thus excluding direct effects of P4 treatment on adenylyl cyclase activity. Moreover, this P4 effect was associated with changes in the binding characteristics and number of CRH-R present in myometrial cells; Scatchard analysis of ^{125}I-oCRH binding in membranes prepared from myometrial smooth muscle cells demonstrated that after P4 pretreatment, CRH binding affinity was increased with apparent Kds of 5.30 ± 2.4 and 1.6 ± 0.9 nM without or with P4 pretreatment, respectively. P4 pretreatment resulted in a 6- to 10-fold increase in the high-affinity binding sites and an increase in the Bmax from 8 ± 5.9 and 65 ± 36 pmol/mg protein.

Because we previously identified that exon 6 plays a major role in determining the response of individual CRH-R1 variants to protein kinase C (PKC) phosphorylation and heterologous desensitization (15), we investigated whether P4 effects on CRH-R1 mRNA expression can alter myometrial CRH-R responses to PKC phosphorylation. After pretreatment with P4 or vehicle for 24 h,

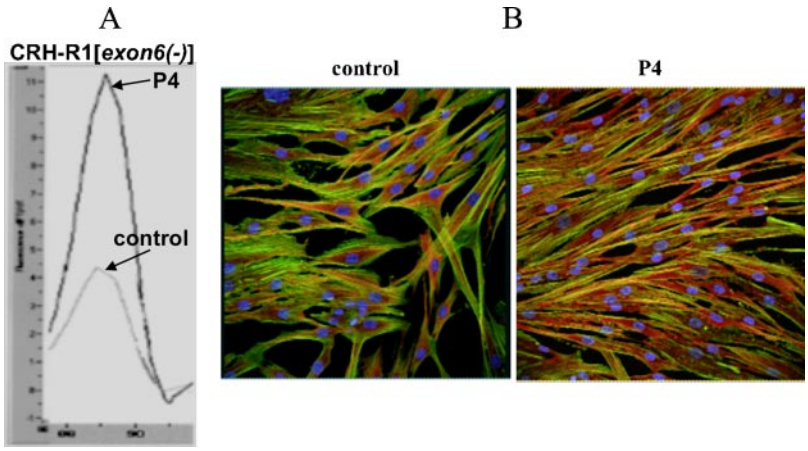

FIG. 5. P4 effects on CRH-R1 exon 6(−) mRNA expression and CRH-R subcellular localization in human myometrial smooth muscle cells. A, Representative maxima of melting curves of CRH-R1 exon 6(−) mRNA transcripts amplified from RNA extracted from cells pretreated with or without 5 μM of P4 for 12 h by real-time quantitative RT-PCR (LightCycler; Roche Diagnostics) as described in *Materials and Methods.* PCR products were electrophoresed on a 1.6% agarose gel. Apart from using the standard curve analysis, quantitation was also carried out, using melting curve analysis. Correction of the amplification curves was carried out by taking a melting curve at the end of the amplification and then calculating the area under the specific product peak, which is related to the amount of product melting at that temperature. B, Effect of 5 μM P4 pretreatment for 12 h on CRH-R immunostaining distribution in human pregnant myometrial cells visualized by indirect immunofluorescence confocal microscopy using specific primary antibodies for CRH-R and Alexa Fluor 594 secondary antibody (*red*) as described in *Materials and Methods*. Distribution of F-actin and stress fibers was also monitored by double staining with Alexa Fluor 488-phalloidin (*green*). Cell nuclei were stained with the DNA-specific dye 4′,6′-diamino-2-phenylindole (*blue*). Identical results were obtained from six independent experiments.

cells were treated for 30 min with or without phorbol 12-myristate 13-acetate (PMA) or the inactive phorbol ester 4α-phorbol-12,13-didecanoate (4α-PDD) followed by addition of 10 μM forskolin or 100 nM CRH for 10 min and estimation of cAMP production. In control cells PMA, but not 4α-PDD, significantly impaired by 40–50% the ability of CRH to stimulate intracellular cAMP production, suggesting that the myometrial cell CRH-Rs were sensitive to PKC activation (Fig. 6A). In contrast, this effect was abolished in cells pretreated with P4. In both types of cells (control or P4 pretreated), basal and forskolin-induced cAMP production was not significantly altered after PMA treatment (data not shown).

Discussion

Critical physiological and pathophysiological steps of pregnancy, such as embryo implantation, fetal immune tolerance, and parturition, are regulated by CRH (21). Expression of CRH-R1 and -R2 receptors in the human myometrium enables CRH and CRH-like peptides to modulate myometrial contractility during pregnancy and labor by exerting distinct and possibly contrasting effects (6, 22). In particular, the CRH-R1 subtype, which mediates the inhibitory effects of CRH on myometrial contractility through activation of the cAMP/adenylyl cyclase and nitric oxide pathways (22, 23), is extensively targeted by alternative pre-mRNA splicing mechanisms that generate a plethora of CRH-R1 splice variants that might contribute in the considerable CRH-R protein heterogeneity observed in human myometrium (24). The expression of these receptor variants, which might act as decoy or dominant-negative receptors (11), is dynamically modulated during pregnancy and labor (17), thus allowing the myometrial tissue to adjust its responsiveness to CRH according to specific requirements during the different stages of pregnancy and labor.

Here we report the identification of a novel myometrial CRH-R1 splice variant (CRH-R1β/d), which has exon 13 removed but retains the R1β-specific exon 6, thus resulting in a final mRNA transcript that is a hybrid between CRH-R1β and -R1d. When overexpressed in HEK-293 cells, the CRH-R1β/d was primarily localized intracellularly and to a lesser degree in the plasma membrane (similar to CRH-R1d) and has reduced binding affinity for CRH (a characteristic of the CRH-R1β). No significant activation of known intracellular signaling pathways was detected, probably due to the impact of combined structural modifications (retention of 29 amino acids in the IC1 and deletion of 14 amino acids from TMD7) that impair CRH-R1 signal transduction. The identification of such a CRH-R1 mRNA variant suggests the presence of specific splicing mechanisms controlling excision or inclusion of exon 13 independently of exon 6 from the final mRNA transcript. These mechanisms appears to be pregnancy specific and active in the early stages of the third trimester of human pregnancy because, similar to our previous studies on CRH-R1d mRNA expression (17), the CRH-R1β/d mRNA was identified in human pregnant (term as well as preterm) but not nonpregnant myometrium. Furthermore, exon 6-containing CRH-R1 mRNA transcripts have been identified in other tissues including the pituitary (8), mast cells (25), and endometrium (26), raising the possibility that CRH-R1β/d mRNA is also expressed in these tissues.

The alternative pre-mRNA splicing mechanisms regulating generation of CRH-R1 variants are unknown. Pisarchik and Slominski (27) reported that in the melanoma cell line SKMEL188, environmental challenges such

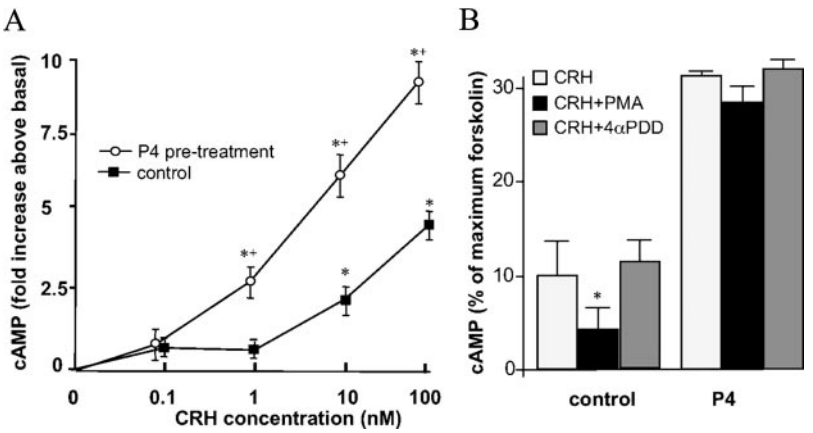

FIG. 6. P4 effects on CRH-induced cAMP response in human myometrial smooth muscle cells and regulation by PKC. A, Effect of P4 on CRH-induced cAMP production from human pregnant myometrial cells. Cells were pretreated with P4, followed by incubation with 0.1–100 nM CRH for 10 min. Results are representative of four independent cell culture preparations. Each *point* is the mean ± SEM of four estimates. *, $P < 0.05$ compared with basal; +, $P < 0.05$ compared with CRH-stimulated values in cells without P4 pretreatment. B, Effects of PKC activation on CRH-induced cAMP production in human myometrial smooth muscle cells pretreated with or without P4. After pretreatment with or without 5 μM of P4 for 12 h, cells were incubated with 200 nM PMA or 500 nM 4α-PDD for 30 min before subsequent stimulation with 100 nM CRH for 10 min and determination of cAMP production as described under *Material and Methods*. Results were normalized by expressed as percentage of forskolin-induced cAMP release, which was considered as maximum (100%) for each group (treated or untreated). Results are presented as the mean ± SEM of five estimations from four individual experiments. *, $P < 0.05$ compared with control (PKC naïve untreated) cells.

as UV irradiation as well as activation of Ser/Thr protein kinases A and C, promote exon 13 retention in the mature CRH-R1 mRNA transcript. Our studies in human primary myometrial smooth muscle cells suggest that estrogens such as E_2 is a potent inducer of exon 13 skipping from the final CRH-R1β mRNA transcript. This result might be physiologically relevant and is in line with our previous studies (17) that demonstrated increased expression of exon 13-negative CRH-R1 mRNA transcripts in laboring (term or preterm) myometrium, which exhibit increased responsiveness to E_2. A major determinant of myometrial sensitivity to actions of estrogens is P4; it has been proposed (28) that P4 prorelaxation effects for most of pregnancy involve inhibition of estrogen receptor-α expression and decreased myometrial sensitivity to estrogens. This would explain why the human myometrium is refractory to the high levels of circulating estrogens for most of pregnancy. At term the functional P4 withdrawal, mediated by an increase in the myometrial PR-A to PR-B expression ratio (29), results in removal of progesterone actions on estrogen receptor-α expression and increases myometrial responsiveness to estrogens that act to transform the myometrium to a procontractile phenotype. This apparent interplay between E_2 and P4 signaling appears also to be important for the control of CRH-R1 splice variant expression because our studies showed that the E2 effect on exon 13 skipping was significantly attenuated in the presence of progesterone. Interestingly, in the primary myometrial cellular model, P4 alone increased expression of exon 6-positive CRH-R1 mRNA variant (primarily CRH-R1β and also CRH-R1β/d) as well as exon 6-negative CRH-R1 mRNA variants such as CRH-R1α, raising the possibility of a different mode of action targeting the *CRHR1* promoter. This possibility is supported by previous studies using *in silico* analysis that identified a progesterone response element within the *CRHR1* promoter region (30); however, more detailed studies are required to confirm this.

It is becoming increasingly evident that CRH exerts an inhibitory effect on contractility of nonlaboring, but not laboring, term myometrium, and this effect involves activation of the CRH-R1 (31, 32). Recently it has been shown that P4 pretreatment can augment CRH actions on myometrial quiescence, suggesting synergistic effects (33). Our studies on the Kd and Bmax binding characteristics of CRH-R suggest that progesterone effects on myometrial CRH-R1 mRNA expression lead to increased amount of high-affinity CRH-R binding sites in the plasma membrane that augment myometrial cell responsiveness to CRH and generation of intracellular signals such as cAMP, which is the main downstream effector in myometrium during pregnancy. In addition, progesterone appears to up-regulate CRH-R1 variants that are resistant to PMA-induced PKC activation. We have previously shown (15) that CRH-R1α and CRH-R1β variants exhibit differential responses to PKC-induced phosphorylation, with only the exon 6-positive CRH-R1β susceptible to signaling desensitization. Taken together, these results suggest that progesterone primarily increases CRH-R1α expression and thus promotes a myometrial CRH-R1 system with reduced sensitivity to heterologous desensitization by PKC. The progesterone-driven regulation of *CRHR1* expression might be active in pregnant nonlaboring myometrium to maintain myometrial quiescence; at term and during labor, P4 effects might diminish due to the functional progesterone withdrawal, and *CRHR1* gene expression levels might be controlled by other contraction-activating signals like IL-1β (17).

In conclusion, we have identified a novel CRH-R1 mRNA variant (CRH-R1β/d) generated by alternative splicing of exon 13 but retaining the R1β-specific exon 6, thus resulting in a final mRNA transcript that is a hybrid between CRH-R1β and -R1d. CRH-R1β/d mRNA is

present in pregnant but not nonpregnant myometrium, and in human primary myometrial smooth muscle cells, its expression is regulated by E_2. The translated protein receptor variant exhibits structural and functional characteristics similar to CRH-R1β and -R1d such as mainly cytoplasmic localization reduced ligand binding affinity and negligible signaling. We also showed that P4 appears to be a potent inducer of exon 6-positive (CRH-R1β) and exon 6-negative (CRH-R1α) CRH-R1 mRNA variants; this leads to increased myometrial cell responsiveness to the actions of CRH. During pregnancy this mechanism might enable the myometrial microenvironment to maintain relaxation.

Acknowledgments

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