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

Eleni Aggelidou, Edward W. Hillhouse, and Dimitris K. Grammatopoulos*

Sir Quinton Hazell Molecular Medicine Research Centre, Department of Biological Sciences, University of Warwick, Gibbet Hill Road, Coventry CV4 7AL, United Kingdom

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The biological actions of corticotropin-releasing hormone (CRH) in the human myometrium during pregnancy and labor are unknown. We hypothesized that CRH may modulate the nitric oxide system, and influence myometrial relaxation/contractility. Incubation of myometrial cells with CRH, but not urocortin II or urocortin III, for 8–16 h significantly induced mRNA and protein expression of endothelial and brain but not inducible nitric oxide synthase (NOS) isoforms. This action resulted in increased activity of soluble guanylate cyclase (GC_s), demonstrated by the enhanced cGMP-producing capacity of the NO donor, sodium nitroprusside. CRH also caused acute activation of the membrane-bound GC, shown by increased basal or atrial natriuretic peptide (ANP)-stimulated cGMP production. These effects appeared to be mediated via the R1 receptors because the CRH receptor antagonists, astressin and antalarmin but not anti-sauvagine 30, could block them. The acute effects of CRH were significantly reduced by inhibition of protein kinase A (PKA) activity, suggesting it is partially PKA dependant. Activation of protein kinase C (PKC) resulted in significant inhibition of both ANP- and CRH-stimulated cGMP production, suggesting a direct effect of PKC on membrane-bound GC. In conclusion, CRH appears to have a dual effect on myometrial NOS/GC pathway, a short term effect predominantly mediated by PKA, and a long-term effect increasing constitutive NOS expression, mediated by a PKA-independent mechanism. This mechanism could potentially be active during human pregnancy, and, because cGMP stimulates myometrial relaxation, these findings further suggest that during pregnancy CRH primarily activates intracellular signals that contribute to the maintenance of myometrial quiescence.

We hypothesized that placentally derived corticotropin-releasing hormone (CRH) plays a central role in coordinating the smooth transition of the uterus from a state of relaxation to one of contraction during labor (1). *In vitro* studies suggest that CRH activates a diverse network of myometrial CRH receptors (CRH-R), expressed differentially during pregnancy (2–4), that are functionally coupled to the adenylate cyclase system only in the pregnant state (5, 6). We have shown that CRH-Rs can activate multiple G-proteins in a tissue-specific manner (7–8) predicting activation of diverse second messenger systems (5, 8–11).

Nitric oxide (NO) may play a role in human uterine contractility, because NO donors decrease the magnitude of oxytocin-induced contractions (12), are used postpartum to relax the uterus for extraction of retained placenta (13), and may suppress uterine contractions in women in preterm labor and prolonged gestation (14). *In vitro* studies favor a role in the maintenance of myometrial quiescence (15–17) and placental vessel tone (18) during human pregnancy. Most, but not all, studies have identified both constitutive and inducible isoforms of NO synthase (NOS) in human uterus (17–20). Furthermore, both constitutive and inducible NOS expression is increased in human myometrium during the third trimester of pregnancy (17, 19) whereas

there is a decline in inducible nitric oxide synthase (iNOS) expression toward term (17) and a reduction in the sensitivity of the myometrium to the NO after the onset of labor.

The actions of NO in the myometrium are mediated, at least partly, via activation of soluble guanylate cyclase (GC_s) and increased production of cGMP, which reduces myosin light chain phosphorylation (21). Myometrial cGMP production can be also regulated by atrial natriuretic peptide (ANP), which activates the membrane-bound guanylate cyclase (GC_m) (22), the cell surface receptor for ANP (23). ANP receptors are widespread within myometrium and other feto-maternal tissues (24) where they possibly mediate ANP's vasodilatory actions on uteroplacental vessels (25). Evidence from myometrial biopsies suggest that the activity of the cGMP/GC system is decreased at the onset of labor (26).

The purpose of this study was to investigate possible interactions between CRH and the NO/cGMP system in the human pregnant myometrium by using primary human pregnant myometrial cell cultures.

Materials and Methods

Experimental Subjects and Preparation of Myometrial Cell Cultures.

Myometrial tissue was obtained from the lower segment of the uterus of women undergoing elective caesarean section for non-maternal reasons at term before the onset of labor ($n = 15$). All patients had no clinical evidence of intrauterine infection. The tissue was immediately processed for myocyte cell culture as described (27). Ethical approval was obtained from the local ethical committee and each patient gave informed consent to the study. The cell cultures were used within 2 weeks of collection.

Synthesis of GTP-AA and Photolabeling of G α Subunits. GTP-AA synthesis and detection of agonist-activated G α subunits in membranes prepared from cultured myometrial cells (100 μ g) were carried out as described (7). The relative density of the bands was measured by optical density scanning by using the software SCION IMAGE-BETA 3B FOR WINDOWS (Scion Corporation, Frederick, MD).

Quantitative Analysis of CRH Receptor Subtypes in Human Pregnant Myometrial Cells. Real-time, one-step PCR for R1 and R2 CRH receptor mRNA (271 and 200 bp, respectively) was performed by using the LightCycler thermal cycler system (Roche Diagnostics)

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Abbreviations: CRH, corticotropin-releasing hormone; NOS, nitric oxide synthase; iNOS, inducible NOS; bNOS, brain NOS; eNOS, endothelial NOS; GC_s, soluble guanylate cyclase; GC_m, membrane-bound guanylate cyclase; ANP, atrial natriuretic peptide; PKA, protein kinase A; PKC, protein kinase C; L-NAME, L-nitroarginine methyl ester; ODQ, 1*H*-[1,2,4]oxadiazolo[4,3- α]quinoxalin-1-one; SNP, sodium nitroprusside; h/rCRH, human/rat CRH.

*To whom reprint requests should be addressed. E-mail: dgrammatopoulos@bio.warwick.ac.uk.

according to the manufacturer's instructions. The primers for CRH-R1 were as follows: sense, 5'-GGCAGCTAGTGGTTCG-GCG-3'; and antisense, 5'-TCGCAGGCACCGATGCTC-3'. The primers for the CRH-R2 were as follows: sense, 5'-CAATGGAGAGGTGCGCTCAG-3'; and antisense, 5'-AA-GAAGGTGGAGGAGGACAG-3'. Negative controls were run in parallel to eliminate possibilities of cross-contamination. To confirm amplification specificity, the PCR products were subjected to a melting curve analysis (data not shown), and 10 μ l of the reaction mixture was subsequently electrophoresed on a 1.6% agarose gel. Quantitative data analysis was made possible through the use of serially diluted known concentrations of CRH-R1 and -R2 mRNAs. The quantification data were analyzed with the LightCycler analysis software (Roche Diagnostics), as described (28).

Immunofluorescent Staining for NOS Isoforms. Immunofluorescence was carried out in myometrial cells as described (27). The primary antibodies for NOS isoforms, (Calbiochem-Nova Biochem) were applied at 1:200 [(anti-brain NOS (bNOS) and -endothelial NOS (eNOS)] and 1:250 (anti-iNOS) dilution in PBS and the secondary anti-rabbit IgG-FITC (Dako) was applied at 1:200. The cell nuclei were visualized by applying the DNA-specific dye 4',6-diamidino-2-phenylindole (DAPI; 1 μ g/ml) for 5 min at room temperature. For negative controls, slides were incubated without the primary antibody, and the results were viewed under a fluorescent microscope by using the appropriate filters.

Immunostaining for NOS Isoforms. Cells were incubated at 37°C for different time intervals (1–16 h) with or without 100 nM human/rat (h/r) CRH (Peninsula Laboratories) or urocortin II or urocortin III (Phoenix Pharmaceuticals, Belmont, CA) in the presence or absence of antalarmin (R1-specific antagonist, 100 nM; ref. 29) or antisauvagine-30 (R2-specific antagonist, 1 μ M; ref. 30) or myristoylated protein kinase A cell-permeable inhibitor fragment 14–22 [10 μ M—a concentration sufficient to inhibit protein kinase A (PKA) activity for at least 12 h] in DMEM. In some experiments, cells were preincubated with IFN- γ (1,000 units/ml) or a mix of IFN- γ (1,000 units/ml) and LPS (30 μ g/ml; Sigma) in DMEM for 16 h. At the end of the incubation period, cells were washed, fixed, and permeabilized and were incubated with antibodies against bNOS, eNOS, or iNOS (1:200 in PBS) overnight at 4°C, rinsed in TBS, and incubated with biotin-conjugated antibody (1:20) for 1 h at room temperature in 1.5% normal goat serum and 0.1% Triton X-100 in TBS. A commercially available extravidin-peroxidase (EXTRA-3) kit (Sigma) was used for visualizing the antibodies. In negative control experiments, cells were incubated with normal goat serum.

Fluorescent *in Situ* Hybridization in Human Myometrial Cells. Cells in DMEM were prestimulated with IFN- γ (1,000 units/ml) for 18 h, followed by treatment with or without h/rCRH (100 nM) for 16 h. Then, cells were rinsed with PBS for 5 min, permeabilized with Pronase E (0.0625 μ g/ml, diluted in PBS), and dehydrated in 100% ethanol. After air drying, 40-mer synthetic oligonucleotide probes (GIBCO), with fluorescein conjugated at their 5' end, in hybridization solution [25% formamide/4 $\times$ SSC/5% dextran sulfate/0.2% dried milk powder (1 nmol/ μ l)] were applied. The slides were left overnight at 37°C, placed in preheated at 45°C 2 $\times$ SSC buffer, and washed for 10–15 min in 0.1 $\times$ SSC. The cell nuclei were visualized as previously described, and the slides were examined under the UV light by using appropriate filters.

The nucleotide sequences, specific for each NOS isoform that was used, are as follows: bNOS (5' to 3'), fluor-GATGGCTGT-GTCTCGAAGTGACGCATGATAGATGTGAAC; eNOS (5' to 3'), fluor-AACTCCAGG(C)₅AATTTCCAGCAGC-ATGTTGGACACTG; iNOS (5' to 3'), fluor-CCTCGTGGCTT-TGGGCTCCTCAAGGCGCTGCCC(T)₆.

Fig. 1. Quantitative analysis of CRH receptor mRNA expressed in human pregnant myometrial tissue and myometrial cells by using the real time PCR. Similar results were obtained from three independent myometrial tissue biopsies/cell preparations.

cGMP Assay. Myocytes reaching 80–90% confluence were incubated at 37°C for 20 min in DMEM with 1 mM IBMX and 1M MgCl₂ (cGMP stimulation buffer). Cells were washed and stimulated with either sodium nitroprusside (SNP) (0.1 mM) or h/rCRH (0–100 nM) and/or ANP (100 nM) and h/rCRH (100 nM) for different periods of time. In some experiments, before agonist stimulation, cells were preincubated with various inhibitors and antagonists—such as L-N^G-nitro-L-arginine methyl ester (L-NAME; 0.1 mM), 1H-[1,2,4]oxadiazolo[4,3- α]quinoxalin-1-one (ODQ; 10 μ M), myristoylated protein kinase A cell-permeable inhibitor fragment 14–22 (10 μ M), astressin (1 μ M), antalarmin (100 nM), anti-sauvagine 30 (1 μ M), phorbol 12-myristate 13-acetate (PMA) (200 nM), or bisindolylmaleimide I (100 nM; Calbiochem-Nova Biochem)—for the required period at 37°C. The reaction was stopped by adding 10 μ l of concentrated HCl. After an overnight freeze/thaw cycle and centrifugation at 600 $\times$ g for 10 min, the cGMP levels in the supernatants were measured by using a commercial ELISA kit (R&D Systems).

Protein Kinase C (PKC) Activity Assay and Immunoblotting of PKC Isoforms. After treatment of myocytes with PMA in the absence or presence of bisindolylmaleimide I (100 nM), PKC activity was determined in whole cell extracts as described (27) by using a nonradioactive PKC assay kit (Calbiochem-Nova Biochem). In addition, after overnight incubation of myocytes with PMA (200 nM), cells were lysed, and proteins were resolved by SDS/PAGE and electrophoretically transferred to nitrocellulose as described (11). Immunoblotting for the PKC isoforms α , β , γ , δ , ϵ , and ζ , was carried out by using specific PKC-isoform antibodies (GIBCO/BRL) at 1:5000 dilution in TBS.

Statistics. The results obtained were expressed as the mean $\pm$ SE. Data were tested for homogeneity, comparison between group means was performed by one- or two-way ANOVA, and probability values of $P < 0.05$ were considered to be significant.

Results

Identification and Quantification of CRH-R mRNA Present in Human Pregnant Myometrial Cells. Both R1 and R2 types of CRH receptors were detected in human pregnant myometrial cells and biopsies.

Fig. 2. NOS isoforms detection by immunofluorescence in human pregnant myometrial cells. Antibodies specific for brain (a and b), endothelial (c and d), and inducible (e and f) NOS isoforms were used in the absence or presence of blocking serum (b, d, and f). Expression of NOS isoforms is indicated by the FITC staining in the cell-cytoplasm. The nuclei were stained blue with 4',6-diamidino-2-phenylindole (DAPI). (Double exposure; magnification, $\times 400$.) Identical results were obtained from three independent myometrial cell preparations.

Serial dilutions of CRH-R1 and R2 genes (100–1 fg) provided the template on which a line of best-fit was plotted and used as a standard curve. Similar amounts of CRH-R1 mRNA were expressed in myometrial tissues and cultured cells. However, myometrial cells expressed at least 50- to 100-fold fewer CRH-R2 gene copies, when compared with the myometrial tissue (Fig. 1).

Effect of CRH on NOS Isoform Expression in Human Pregnant Myometrial Cells. Immunofluorescent staining confirmed the presence of both types of constitutive (brain and endothelial) NOS (Fig. 2 a and b, c and d) and iNOS (Fig. 2 e and f). The intensity of iNOS staining, however, was qualitatively less than that in cells stained for eNOS and bNOS. Stimulation with IFN- γ (1,000 units/ml) or LPS (30 μ g/ml) caused a small, but significant, increase in iNOS expression (data not shown). The same cells incubated with normal serum without primary antibody showed no evidence of staining.

CRH (100 nM) increased expression of both eNOS and bNOS protein levels after 8 h incubation with a maximal effect at 16 h (Fig. 3 a and b). In contrast, CRH treatment had no effect on iNOS expression (Fig. 3 c and d). Incubation of myometrial cells for up to 24 h with 100 nM urocortin II or urocortin III (type 2 CRH receptor specific agonists) failed to modulate cNOS or iNOS expression (data not shown). Antalarmin (100 nM) (Fig. 3i) but not anti-sauvagine 30 (1 μ M; Fig. 3j) blocked the CRH effect whereas PKA inhibition had no effect on CRH activation of cNOS (data not shown). In addition, by using fluorescent *in situ* hybridization to localize the NOS mRNA cellular distribution, we showed that, after

8–16 h, CRH (100 nM) significantly increased the fluorescent staining, suggesting increased expression of the mRNAs encoding the eNOS and bNOS (Fig. 3 e and f). In contrast, CRH (100 nM) for up to 24 h had no effect on basal or IFN- γ -stimulated iNOS mRNA expression (Fig. 3 g and h).

Myometrial NOS Up-Regulation by CRH and Downstream GC $_s$ Activation. In the myometrial cells, a significant level of basal GC activity was found, as measured by cGMP production (0.022 ± 0.0042 pmol/ml). Furthermore, SNP (0.1 mM) caused a rapid increase in cGMP production, with maximal stimulation at 5–10 min (4.5 ± 0.3 -fold above basal) and return to basal levels within 60 min. To determine whether CRH-induced up-regulation of NOS expression might alter the activity of NOS-GC $_s$, cells were preincubated with various concentrations of CRH for 16 h. This treatment resulted in a dose-dependant augmentation of SNP-induced cGMP stimulation at a concentration range of 10–100 nM CRH (6.6 ± 1.1 vs. 3.9 ± 0.3 -fold above basal with or without 100 nM CRH pretreatment; Fig. 4a). Furthermore, CRH pretreatment caused a small but significant increase in basal cGMP production (1.7 ± 0.6 -fold above basal at 100 nM CRH pretreatment). This effect of CRH was attenuated by L-nitroarginine methyl ester (L-NAME; 0.1 mM), a NOS inhibitor, but not by a specific PKA inhibitor (Fig. 4 b and c). Importantly, the CRH receptor antagonists astressin (1 μ M) and antalarmin (100 nM) but not anti-sauvagine 30 (1 μ M; Fig. 4c) blocked the CRH effect, suggesting the involvement of R1 receptors. These agents had no effect on SNP-induced cGMP production.

Acute effects of CRH on the NOS-GC $_s$ system activity, without involving NOS up-regulation, were evaluated by addition of CRH for 5 min in the presence of SNP; this treatment resulted in increased basal or SNP-stimulated cGMP levels (Fig. 5a). Because the acute effect of CRH could be due to activation of NOS and/or simultaneous activation of GC $_s$ or GC $_m$, we used specific inhibitors and investigated the effect of CRH on basal or SNP-stimulated cGMP production. Preincubation with L-NAME did not affect the SNP nor the CRH effect on basal (SNP untreated) and SNP-induced cGMP production, suggesting that the CRH effect on cGMP production was due to direct stimulation of either GC $_m$ or GC $_s$. This result was tested by preincubating the cells with a specific GC $_s$ inhibitor, ODQ (10 μ M), for 30 min before CRH or SNP addition. This treatment abolished SNP-stimulated cGMP increase and caused a small but significant reduction (20%) in CRH-stimulated basal or SNP-stimulated cGMP production, suggesting that the acute CRH

Fig. 3. Changes in myometrial cell NOS isoform protein or mRNA expression or detected by immunostaining or fluorescent *in situ* hybridization (FISH). (a–d, i, and j) Cells treated without or with CRH for 16 h before staining for eNOS (a and b) or iNOS (e and f) with specific antibodies. Also, cells were treated without or with CRH for 16 h in the presence of antalarmin (100 nM; i) or anti-sauvagine 30 (1 μ M; j). (e–h) Cells treated without or with CRH for 16 h before staining for eNOS (e and f) or iNOS (g and h) with specific oligonucleotide probes. (Magnification, $\times 400$.) Identical results were obtained from five independent cell preparations.

Fig. 4. Effect of CRH pretreatment on GC_s activity in human pregnant myometrial cells. cGMP levels of cells pretreated for 16 h with (a) different concentrations of CRH or (b) 100 nM CRH in the presence or absence of astressin (1 μM), or (c) 100 nM CRH in the presence or absence of myristoylated protein kinase A inhibitor fragment 14–22 (10 μM) or antalarmin (100 nM) or anti-sauvagine 30 (1 μM), before stimulation with SNP (0.1 mM) with or without L-NAME (0.1 mM). Results are representative of three independent cell preparations, and each point is the mean $\pm$ SE of three determinations. *, $P < 0.05$, compared with basal values; +, $P < 0.05$, compared with SNP-stimulated cGMP values.

effect on cGMP production was due to direct stimulation of GC_m with a small contribution from direct activation of GC_s .

CRH-Induced G-Protein Activation in Human Pregnant Myometrial Cells. CRH, acting via adenylate cyclase, can activate PKA. Because the CRH effect on myometrial NOS expression was not affected by PKA inhibition, we investigated whether CRH may act via alternative pathways by characterizing the G-protein subtypes activated by CRH in myometrial cells. We found that the CRH receptors are coupled to at least five G-proteins: Gs, Gq/11, Gil/2, Go, and Gz (Fig. 5b). At the highest concentration tested (100 nM), CRH was most potent in activating Gs- and Go-protein subtypes (5.2 ± 0.5 and 4.8 ± 1.5 -fold above basal, respectively), and least potent on activation of Gz-protein (1.9 ± 0.3 -fold above basal).

Effect of CRH on Myometrial GC_m Activity. Our previous experiments suggested interactions between CRH and the membrane-bound component of myometrial GC. We established the functional integrity of GC_m in our myometrial cells by measuring ANP-induced cGMP production. ANP (100 nM) increased cGMP levels in a time-dependent manner, with maximal cGMP accumulation after 5 min (9.5 ± 3.3 -fold above basal). This response was sustained for at least 60 min and was 150–300% greater than that elicited by SNP, suggesting that, in myometrial cells, the GC_m is mainly responsible for the generation of cGMP.

Cells stimulated with CRH increased cGMP production in a time-dependent manner (maximal cGMP accumulation after 5–10 min, 4.5 ± 1.1 -fold above basal) and dose-dependent

Fig. 5. (a) Acute CRH effects on GC_s activity in human pregnant myometrial cells. cGMP levels of cells pretreated for 30 min with vehicle alone, L-NAME (0.1 mM), or ODQ (10 μM) before stimulation with SNP (0.1 mM) in the presence or absence of 100 nM CRH. Results are representative of three independent cell preparations, and each point is the mean $\pm$ SE of three determinations. *, $P < 0.05$, compared with basal values; +, $P < 0.05$, compared with SNP-stimulated cGMP values. **, $P < 0.05$, compared with values obtained from cells pretreated with vehicle alone followed by stimulation with CRH or CRH + SNP. (b) Autoradiograph of CRH-induced photolabeling (with [32 P]GTP-AA) of various Gα-proteins in human pregnant myometrial cells. Identical results were obtained from six independent cell culture preparations.

manner, with a threshold of 10 nM and maximal increase (3.5 ± 0.84 -fold above basal) at the highest concentration (100 nM) tested (Fig. 6a). Furthermore, CRH had a synergistic effect on ANP-induced cGMP stimulation (Fig. 6b). This effect of CRH was dose dependent, with a threshold of 10 nM and maximal increase at a concentration of 100 nM (3.33 ± 1.3 -fold above ANP-induced stimulation). This phenomenon was detectable at 5–10 min of incubation, disappeared within 30 min (data not shown) and was blocked by the CRH-R antagonists astressin (1 μM) and antalarmin (100 nM) but not anti-sauvagine-30 (1 μM; Fig. 6b). However, L-NAME did not inhibit (data not shown), whereas ODQ caused a small but significant decrease (18%) on the CRH-effect on basal or ANP-stimulated cGMP production (Fig. 6c), suggesting a small contribution from activation of the GC_s . In addition, inhibition of myometrial PKA activity by the myristoylated protein kinase A inhibitor fragment 14–22 significantly reduced by 50% the CRH effect on basal or ANP-induced cGMP release (Fig. 6c). Similar results were obtained with forskolin (10 μM; a direct activator of PKA; 2.13 ± 0.8 -fold above ANP-induced stimulation); ODQ caused a similar to CRH decrease (22%) on the forskolin-effect on basal or ANP-stimulated cGMP production. However, addition of the protein kinase A inhibitor completely abolished the forskolin effect on ANP-induced cGMP release (Fig. 6c).

Effect of PKC Activation on Myometrial GC_m and GC_s Activity. The effect of PKC activation on CRH-NOS/ GC_s and GC_m interactions was determined by using PMA as a PKC activator. Incubation of myometrial cells with 200 nM PMA for 30 min had no effect on SNP-induced or CRH + SNP-induced cGMP production (Fig. 7a). Similarly, the CRH-induced NOS up-regulation was not affected by PMA (data not shown). Down-regulation of PKC (by overnight incubation of myometrial cells with PMA) or inhibition of PKC activity by bisindolylmaleimide I (100 nM for 2 h) also had no effect on SNP alone or CRH + SNP-induced cGMP production, suggesting that PKC under basal or PMA-stimulated conditions cannot influence this pathway. In contrast, pretreatment of cells with PMA reduced the effect of CRH-, ANP-, or ANP plus CRH-induced

Fig. 6. Acute CRH effects on GC_m activity in human pregnant myometrial cells. cGMP levels of cells incubated with (a) different concentrations of CRH, or (b) ANP (100 nM) plus CRH (0–100 nM; filled diamonds) in the absence or presence of antalarmin (100 nM; filled squares), or anti-sauvagine 30 (1 μM; open squares) or astressin (1 μM; open circles). (c) Cells preincubated for 30 min in the presence or absence of myristoylated protein kinase A inhibitor fragment 14–22 (10 μM), or ODQ (10 μM) before stimulation with ANP (100 nM), CRH (100 nM), forskolin (10 μM), or ANP (100 nM) plus either CRH (100 nM) or forskolin (10 μM). Results are representative of three independent cell culture preparations, and each point is the mean ± SE of three determinations. *, $P < 0.05$, compared with basal values; +, $P < 0.05$, compared with ANP-stimulated cGMP values. **, $P < 0.05$, compared with values obtained from cells pretreated with vehicle alone followed by stimulation with CRH (or forskolin) + ANP.

stimulation of cGMP production significantly by $48 \pm 5\%$, $60 \pm 8\%$, and $83 \pm 10\%$, respectively (Fig. 7b), suggesting an inhibitory action of PKC on myometrial GC_m. This effect of PMA could be blocked by inhibition of PKC activity by bisindolylmaleimide I or by down-regulation of PKC (by overnight incubation of myometrial cells with PMA; Fig. 7b). Both treatments caused a significant inhibition of PKC activity ($73 \pm 5\%$ and $62 \pm 7\%$, respectively; Fig. 7c). In addition, by using immunoblotting with PKC-isoform specific antisera, we showed that overnight PMA treatment primarily down-regulated the expression of all major PKC isoforms expressed in myometrial cells, namely α , β , and ζ . (Trace amounts of PKC ϵ were also detected, but its expression did not appear to be altered by overnight PMA treatment Fig. 7c). Treatment with bisindolylmaleimide I or PMA overnight had no effect on CRH- or ANP-induced cGMP production (data not shown).

Discussion

This study demonstrates a clear functional link between CRH and myometrial GC activation and subsequent generation of intracellular cGMP in human pregnant myometrial cells. Clifton *et al.* (10) provided the first evidence for such a link in the human

Fig. 7. Effect of phorbol 12-myristate 13-acetate (PMA) on GC_s and GC_m activity in human pregnant myometrial cells. cGMP levels of cells preincubated with either PMA overnight or for 2 h with or without bisindolylmaleimide I (100 nM) before 2-h treatment with or without PMA (200 nM). Subsequently, cells were stimulated with (a) SNP (0.1 mM) or (b) ANP (100 nM), or ANP (100 nM) plus CRH (100 nM). The effects of overnight PMA or 2 h with bisindolylmaleimide I treatment on PKC activity or PKC isoform expression are shown in c. Results are representative of three independent cell preparations, and each point is the mean ± SE of three determinations. *, $P < 0.05$, compared with untreated values; +, $P < 0.05$, compared with ANP or ANP plus CRH-induced cGMP values in the absence of PMA.

fetal-placental circulation, where CRH stimulates vasodilation via a nitric oxide-cGMP-dependent pathway (10). Similar findings have been reported in the pituitary (31). This interaction involves two distinct signaling cascades, one of which is PKA independent and involves prolonged stimulation by CRH and up-regulation of constitutive (endothelial and brain) NOSs and activation of GC_s. The other requires acute stimulation by CRH, is partially PKA dependent, and enhances GC_m and to a lesser degree GC_s activity, resulting in potentiation of the ANP- and SNP-induced cGMP production.

Although myometrial cells express mRNA for R1 and R2 receptor, the CRH effects on NOS and GC appear to be mediated exclusively via the R1 receptor subtype, as shown by the use of specific receptor antagonists. In addition, specific agonists of R2 receptors such as urocortin II and III failed to influence NOS expression and GC activity. One possible explanation for this result is the low expression of CRH-R2 receptors in these cells, which is suggested by our quantitative mRNA expression data. However, we cannot exclude the possibility that, in tissues where R2 receptor subtypes are expressed in abundance, CRH and the family of urocortin peptides may be able to modulate the NOS/GC system. In support of the latter it has been recently shown that, in H5V cells that express both types 1 and 2 CRH-Rs, CRH inhibited cytokine-induced iNOS expression, an effect mediated via CRH-R1 receptors, whereas, in HUVEC cells that express only CRH-R2, CRH potentiated cytokine-induced iNOS expression (32).

Our data suggest that brain, endothelial, and inducible NOS isoforms are present in the smooth muscle cells in myometrium at term. Several investigators have demonstrated increased myometrial cNOS expression during human pregnancy (19), and increased levels of CRH during the third trimester (33) may play a role in coordinating this expression. Signaling pathways such as the p38 and extracellular signal-regulated kinase (ERK) cascade can activate the human iNOS promoter (34), and the fact that CRH cannot activate the ERK cascade in pregnant myometrial

cells (12) might explain the differential effects on cNOS and iNOS expression. It would be of interest to investigate the effects of urocortin, an activator of ERK in myometrial cells (12), on the regulation of cNOS and iNOS expression and GC activity.

The myometrial NO-GC_s-cGMP system can be activated by endogenous peptides, such as the calcitonin gene-related peptide, to promote relaxation and inhibit contractions (17). Our data suggest that prolonged treatment of human pregnant myometrial cells with CRH can augment the GC_s activity indirectly via up-regulation of cNOS expression, an effect that appeared to be independent of the PKA pathway. However, cAMP can modulate other signaling cascades, including protein kinase B, small GTPases, and subsequently mitogen-activated protein kinases (MAPKs), in a PKA-independent fashion (35); the possibility thus remains that similar cAMP-dependent but non-PKA-mediated mechanisms are responsible. In addition, other pathways might be involved because in these cells stimulation of endogenous CRH receptors leads to activation of multiple G-protein subtypes with an order of potency Gs≥Go>Gq₁₁>Gi_{1/2}>Gz. It should be emphasized that, for some of these G-proteins, the precise second messenger system activated is unknown.

The membrane-bound form of guanylate cyclase (GC_m) also regulates myometrial cGMP production and contractility (22). CRH acutely increased basal and ANP-stimulated GC_m activity, an effect that appeared to be independent of NOS activity. The CRH-GC_m cross-talk is partially dependent on PKA, and it has been shown that phosphorylation of the kinase homology domain is essential for activation of the GC_m (36). It is also possible that PKA action is required for a direct functional link between the CRH-R and GC_m without involving PKA-induced phosphorylation of GC_m. In addition, the PKA inhibitor failed to block completely the CRH effect, which raises the possibility of additional intracellular components mediating the CRH-GC_m interaction.

In human pregnancy at term, the action of uterotons such as oxytocin is mediated via activation of protein kinase C. In our system, activation of PKC was found to inhibit the effect of CRH selectively on the GC_m but not NOS-GC_s activity. Interestingly, several investigators have reported both stimulatory (37) and inhibitory actions of PKC on GC_m activity mediated by dephosphorylation of specific residues (38). Possibly, this result depends on specific PKC-isoforms; the myometrial cells appear to contain α , β , ζ , and trace amounts of PKC ϵ . In addition, the inhibitory effect of PMA on CRH-induced cGMP elevation cannot be attributed solely to the effect on GC_m activity because we have previously reported that PKC can reduce the biological activity of the human myometrial CRH receptor (27). Interestingly, in pituitary cells, PKC can exert a different action on CRH signaling characteristics and potentiates CRH-stimulated cAMP production (39, 40). It is likely that, in the myometrial cells, PKC targets multiple components of the intracellular pathway to reduce the CRH-induced cGMP response.

In conclusion, CRH acting via type 1 CRH receptors in human myometrial cells, appears to exert a dual effect on the NOS/cGMP system, increasing cNOS expression, mediated via a PKA-independent mechanism, and acutely activating GC_m and GC_s via a PKA-dependant mechanism. The latter can be blocked by PKC, an important mediator of uterotons effects on myometrial contractility. These data further reinforce our hypothesis that CRH plays a major role in the maintenance of myometrial quiescence during pregnancy.

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