Evidence that Corticotropin-Releasing Hormone Modulates Myometrial Contractility during Human Pregnancy

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As human pregnancy advances, CRH increases exponentially and is hypothesized to trigger the transition from myometrial quiescence to active contractions at labor. Paradoxically, CRH stimulates cAMP production, suggesting it should cause relaxation. To evaluate CRH as a mediator of quiescence, the effect of CRH on contractions in preterm and term myometria with concurrent progesterone (P4) was determined. In late gestation, we hypothesized that high concentrations of CRH down-regulate agonist-activated-cAMP relaxatory pathways and that increased phosphodiesterase (PDE) activity induces heterologous down-regulation of agonist-activated-cAMP pathways. CRH caused dose-dependent relaxation of spontaneously contracting myometrial strips of 31 ± 8% (mean ± SEM; n = 12) and 35 ± 20% (n = 3) in term and preterm samples, respectively. CRH with P4 pretreatment caused a 40 ± 13% (n = 4) reduction in contractility, whereas in matched samples, CRH alone exerted a 26 ± 6% (n = 4) reduction, with a shift of CRH dose-response curves ($P < 0.01$, ANOVA). Pretreatment of strips with 10^{-7} M CRH did not attenuate relaxation induced by subsequent CRH (n = 3) or salbutamol (β_2-agonist) treatment (n = 9). PDE inhibition by rolipram showed a 2.2- and 1.5-fold increase in maximal relaxation induced by CRH and salbutamol, respectively, with a shift of both dose-response curves ($P < 0.05$ and $P < 0.01$, ANOVA). In conclusion, CRH at physiological concentrations acts synergistically with P4 contributing to myometrial quiescence. P4 withdrawal may reduce CRH-mediated relaxation. Our functional model does not support homologous or heterologous down-regulation of agonist-stimulated-cAMP pathways by high CRH concentrations. PDE inhibition potentiates CRH and salbutamol-induced relaxation. Up-regulation of PDEs, through chronic cAMP elevation by CRH, could provide a mechanism for down-regulation of agonist-stimulated-cAMP pathways at term. **(*Endocrinology* 150: 5617–5625, 2009)**

Human pregnancy is characterized by a progressive increase in placental production of the peptide hormone CRH, with fetal plasma levels at term of 100–250 pg/ml and maternal levels typically exceeding 1000 pg/ml (1, 2). Maternal plasma levels rise from the second trimester, increasing dramatically in the final 5–6 wk before delivery with a rapid drop postpartum (3, 4). Despite this distinctive pattern, the precise biological function of CRH during gestation and parturition remains unknown, and several roles are proposed (5). For example, CRH is hypothesized to promote fetal maturation (6, 7), stimulate dehydroepiandrosterone sulfate production in the fetal adrenal (8) and contribute to dilatation of the placental vasculature (9). Its function in the maternal compartment is less clear, but CRH may be an important regulator of myometrial contractility.

The myometrium is strongly implicated as a physiological target for CRH, expressing a multitude of CRH receptors (CRHR), which are dynamically regulated (10–12) and exhibit higher affinity for CRH during pregnancy (13). CRHR1α variant is considered a functional receptor, with efficient CRH-mediated cAMP production, suggesting coupling to Gα_s/adenylate cyclase (AC) (14). CRH stimulation of pregnant myometria *in vitro* increases

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Abbreviations: AC, Adenylate cyclase; CRHR, CRH receptor; GPCR, G protein-coupled receptor; GKR, GPCR kinase; NL, nonlaboring; P4, progesterone; PDE, phosphodiesterase; PKA, protein kinase A; β_2-R, β_2-adrenergic receptor.

cAMP, suggesting CRH should cause uterine relaxation (14, 15). However, contractility studies report conflicting data. Early studies observed CRH potentiation of oxytocin or prostaglandin $F_{2\alpha}$-stimulated contractions (16–18). These studies examined electrically stimulated myometrial strips (18) or strips displaying no spontaneous contractions (16, 17) and thus may not represent the *in vivo* state, given that myometrium is spontaneously active and contracts without hormonal or neural stimulation (19). Subsequent studies using spontaneously contracting myometria report that CRH exerts no effect on nonlaboring (NL) term tissue (20), decreases the duration of the plateau phase of relaxation (21), and causes a reduction in contraction amplitude in NL samples, with reduced CRH responsiveness at labor (22).

Biochemical studies suggest the ability of CRH to activate CRHR1α/Gα_s/AC/cAMP pathways is less effective with advancing gestation (14). This may be due to receptor or postreceptor mechanisms, which act to limit CRH-induced myometrial relaxation in late term or parturition. This hypothesis has not yet been confirmed with functional studies. Receptor-based mechanisms could include long-term down-regulation of CRHR1 (23) and reduced coupling to Gα_s, partly due to decreased expression of the latter in laboring myometria (24). *In vitro* studies suggest high levels of CRH acutely desensitize CRHR1 (25–27). Pregnant myometrial cells treated with CRH demonstrate impaired cAMP production to a second CRH challenge (27). Teli *et al.* (27), employing HEK cells overexpressing CRHR1α, showed that high-dose CRH pretreatment resulted in phosphorylation, internalization, and decreased coupling of CRHR1α to Gα_s. G protein-coupled receptor (GPCR) kinases (GRKs) 3 and 6 mediated this homologous desensitization of CRHR1α (27). GRK 6 is also implicated in homologous desensitization of rat myometrial β_2-adrenergic receptors (β_2-Rs) (28, 29). It is possible that high concentrations of CRH, as found at term, lead not only to homologous desensitization of CRHRs (27), but also to heterologous desensitization of other GPCR such as the β_2-R. Thus, CRH could play a central role in the onset of labor, directly diminishing the efficacy of myometrial relaxatory pathways, leading to an increase in contractility at term.

Postreceptor mechanisms contributing to impaired agonist-induced cAMP production at term could include increased cAMP degradation. cAMP is hydrolyzed by members of the cyclic nucleotide phosphodiesterase (PDE) superfamily. Of the five PDE isoforms identified in human pregnant myometrium, PDE4 is dominant (30). PDE4 activity is increased in late gestation, accounting for 75% of total cAMP hydrolytic activity (31). Inhibition of myometrial-specific PDE isoforms would thus increase cAMP content and enhance relaxation. Rolipram, a selective PDE4 inhibitor, is an effective relaxant of pregnant myometrial strips (31). Progesterone (P4) may also cause PDE inhibition (32, 33).

Our studies examined the functional effect of CRH on spontaneous myometrial contractions *in vitro*. Specifically, we hypothesized that 1) CRH-induced relaxation is greater in preterm than in term myometrial tissue, 2) the effect of CRH is enhanced with concurrent P4 or with concurrent PDE inhibition, 3) high levels of CRH lead to homologous desensitization of CRHR with attenuation of CRH-induced relaxation, and 4) high levels of CRH lead to heterologous desensitization of the β_2-R with attenuation of β_2-agonist-induced relaxation.

Materials and Methods

Subjects

All experiments performed were approved by the Hunter Area Research Ethics Committee, adhering to the guidelines of University of Newcastle and John Hunter Hospital. Nonlaboring human myometrial samples ($5 \times 5 \times 10$ mm) were obtained from the upper edge of the lower uterine segment at cesarean section. Immediately after biopsy, samples were dissected from connective tissue and placed into ice-cold saline. Samples were stored at 4 C, and contractility experiments were performed within 16 h of collection. In the term group, maternal age was 31.6 ± 1.2 yr (mean ± SEM), with gestational age 38 wk 5 d ± 6 d (mean ± SEM). Indications for section were previous section (n = 19), breech (n = 3), placenta praevia (n = 1), poor progression (n = 1), previous myomectomy (n = 1), and ovarian cyst (n = 1). For the preterm group, maternal age was 21–42 yr, with gestational age ranging from 34 wk to 36 wk 1 d. Sections were performed for placenta praevia (n = 2) and scar dehiscence (n = 1).

Isometric tension recordings

Samples were cut into strips ($7 \times 2 \times 2$ mm) and suspended in organ baths containing 15 ml Krebs-Henseleit buffer, supplemented with 1.89 mM $CaCl_2$. Strips were connected to a Grass FT03C force transducer (Grass Instruments, Quincy, MA) and 1 g passive tension applied. Buffer was replaced five times during the first hour. Strips were maintained at 37 C/pH 7.4 and continuously bubbled with 95% O_2/5% CO_2. Strips were equilibrated a further 60–90 min, until regular spontaneous contractions developed. Strips generating peak contractions of less than 1 g were discarded. Data were digitized using a Maclab8E data-acquisition system and analyzed using Chart software (ADI, Melbourne, Australia). Contractility was measured as the integrated area under the tension-time curve. Response was reported as a percentage of spontaneous baseline activity before any treatment. Complete inhibition of contraction was defined as 100% relaxation from basal levels. Strips were exposed to cumulative log doses of investigational drugs typically at approximately 20-min intervals. The CRH dose range (10^{-12} to 10^{-6} M) was based on studies of CRH-stimulated cAMP production in pregnant myometrial membrane extracts (14). Untreated and vehicle controls were included in each experiment as appropriate.

Materials

CRH was obtained from Auspep (Parkville, Australia). The β_2-adrenergic agonist salbutamol was obtained from Allen and Hanburys (Middlesex, UK). Forskolin, rolipram, and P4 (4-pregnene 3,20-dione) were purchased from Sigma-Aldrich (St. Louis, MO). Fresh Krebs-Henseleit buffer (120 mM NaCl, 4.7 mM KCl, 1.0 mM $MgSO_4$, 1.0 mM NaH_2PO_4, 10 mM glucose, 25 mM Na_2HCO_3) with 1.89 mM $CaCl_2$ was made daily.

Data analysis

Dose-response curves were constructed using integrated area per 10-min intervals. Data are expressed as the mean ± SEM. Maximal effect is reported as the maximal observed effect in the range of concentrations tested. EC_{50} was calculated after fitting of idealized dose-response curves using GraphPad Prism software (San Diego, CA). Curves were compared by nonlinear regression analysis, using GraphPad Prism. ANOVA for multiple comparisons was performed. Significance was accepted for $P < 0.05$.

Results

CRH induces relaxation in term and preterm myometria *in vitro*

Spontaneously contracting preterm and term myometrial strips were treated with cumulative doses of CRH from 10^{-12} to 10^{-6} M (Fig. 1). In term myometria, CRH caused a modest dose-dependent reduction in contractility, with a mean maximal inhibition of 31 ± 8% (mean ± SEM, n = 10–12) (Fig. 2A). In preterm myometria, CRH induced a similar mean maximal reduction of 35 ± 20% (n = 3). There was no displacement of dose-response curves (ANOVA, $P > 0.5$). Maximal relaxation response to CRH in myometrial strips was highly variable with no correlation with gestational age (Fig. 2B). The potency of CRH was unchanged in term and preterm samples (Fig. 2C). Forskolin, an AC activator, produced more consistent relaxation, with an 88 ± 7% (mean ± SEM, n = 6–9) reduction in contractility, with a 2.8-fold greater maximal inhibition of contraction than CRH (Fig. 2D).

CRH-induced relaxation in term myometria is enhanced by P4

Because high circulating concentrations of P4 are characteristic of pregnancy *in vivo*, the contractile response to CRH was determined in the presence of P4 (Fig. 3A). In term myometria, after P4 pretreatment (10^{-8} M, 60 min), the CRH dose-response curve was significantly displaced (ANOVA, $P < 0.01$) with increased efficacy of CRH (Fig. 3B). In this series, CRH alone caused a maximal 26 ± 6% reduction in contractility (mean ± SEM, n = 4–6). In matched samples, cumulative doses of CRH in the presence of P4 resulted in a 40 ± 13% (n = 4–6) reduction in

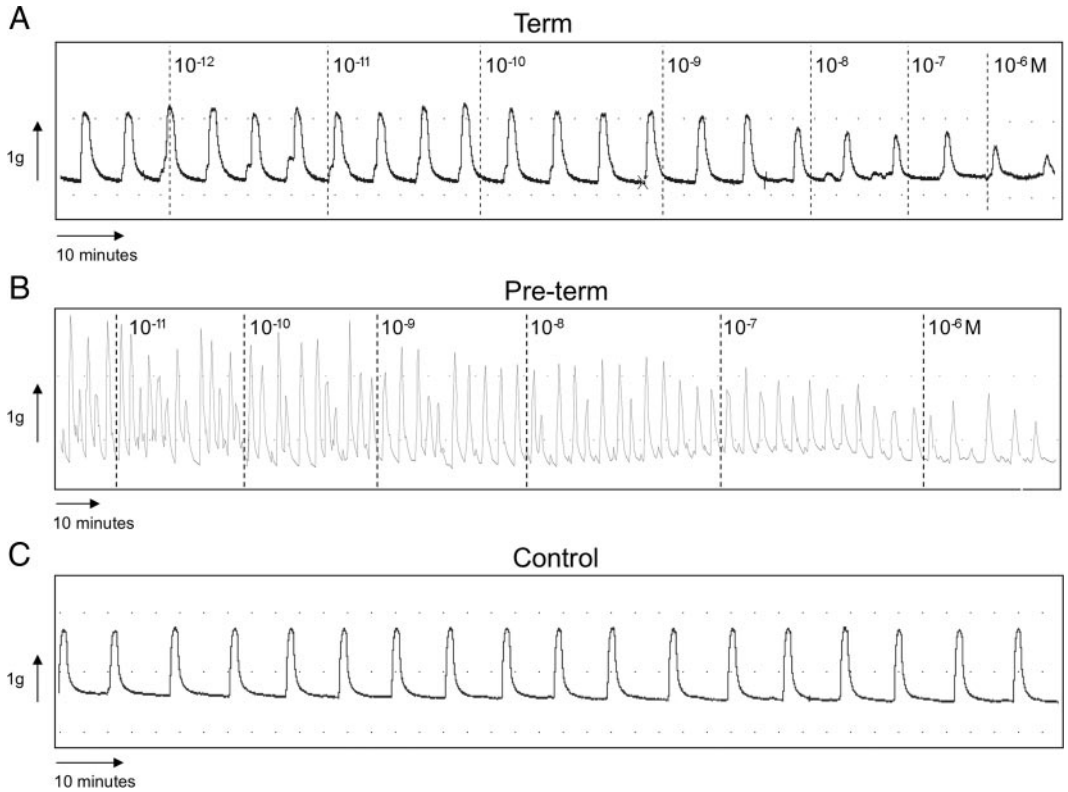

FIG. 1. CRH-induced relaxation in term and preterm myometria. Representative isometric tension recordings of spontaneous contractions in human myometrial strips after cumulative doses of CRH 10^{-12} to 10^{-6} M (term) (A), CRH 10^{-11} to 10^{-6} M (preterm group) (B), or untreated control (C). Tension generated is represented in grams, with time in minutes.

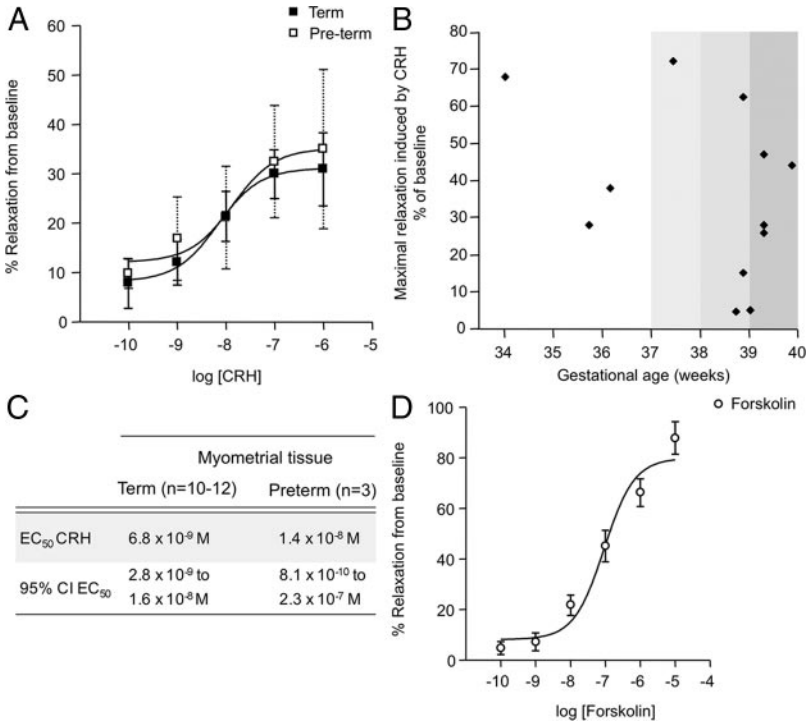

FIG. 2. Effect of CRH on spontaneous myometrial contractility. A, Cumulative effect of CRH on contractile activity. Relaxation response to treatment was compared as a percentage of basal activity before treatment. Data points represent the mean ± SEM. Relaxation was measured as integrated area under the tension curve. B, Maximal observed effect of CRH in relation to gestational age. Individual data points are shown. C, Potency of CRH. EC_{50} was calculated after fitting of idealized dose-response curves using GraphPad Prism software. CI, Confidence interval. D, Cumulative effect of forskolin on contractile activity. Data points represent the mean ± SEM.

contractility. The potency of CRH was unchanged by P4, with EC_{50} 1.2×10^{-8} and 8.9×10^{-9} M in the presence and absence of P4, respectively ($P > 0.05$, 95% confidence interval 4.5×10^{-10} to 3.0×10^{-7} M with P4 and 1.4×10^{-9} to 5.6×10^{-8} M without P4). At 10^{-9} M CRH, consistent with physiological concentrations at term, relaxation increased 3.5-fold in the presence of P4. In preterm myometria, P4 pretreatment did not enhance CRH-induced relaxation (Fig. 3C). Treatment with P4 at 10^{-8} M alone exerted no significant change in contractility from baseline, with a 7 ± 3% reduction (n = 9). Similarly, in control strips, there was no change in tension, with a 6 ± 4% (n = 15) reduction from baseline at the conclusion of the experiments (data not shown).

CRH-induced relaxation in term myometria is enhanced by rolipram

The PDE type 4B inhibitor rolipram inhibits cAMP degradation and therefore should potentiate cAMP. The effects of CRH or salbutamol on contractility were examined in the presence and absence of rolipram (Fig. 4A). In these studies in term myometrial strips, CRH caused a maximal 18 ± 6% reduction in contractility (mean ± SEM, n = 4), whereas salbutamol produced maximal relaxation of 22 ± 6% (n = 5–6) (Fig. 4, B and C). In matched strips pretreated with rolipram, CRH caused a maximal relaxatory response of 40 ± 6% (n = 4). The dose-response curves were significantly displaced, with an enhanced effect of CRH in the presence of rolipram ($P < 0.05$, ANOVA), with a 2.2-fold increase in maximal response (Fig. 4B). The potency of CRH was unchanged by rolipram, with EC_{50} 1.5×10^{-8} and 8.7×10^{-9} M in the presence and absence of rolipram, respectively ($P > 0.05$). Similarly, salbutamol in the presence of rolipram produced a maximal relaxatory response of 33 ± 6% (n = 5–6) in matched myometrial strips (Fig. 4C). The salbutamol dose-response curves were significantly displaced ($P < 0.01$, ANOVA), with a 1.5-fold increase in the maximal response to salbutamol after rolipram pretreatment. The EC_{50} of salbutamol (1.4×10^{-7} M) was unchanged by rolipram (1.5×10^{-8} M). Rolipram 10^{-9} M alone caused 9 ± 2% relaxation (n = 10), with untreated control strips showing a 6 ± 7% (n = 11) reduction from baseline at the conclusion of the experiments (data not shown).

Contractile response to CRH is unchanged in the presence of high-dose CRH

To determine whether high concentrations of CRH cause desensitization of the CRHR, with attenuation of subsequent CRH-induced relaxation, the effect of CRH on contractility was examined in the presence of CRH pretreatment. Matched spontaneously contracting strips were either 1) treated with CRH 10^{-12} to 10^{-6} M or 2) pretreated with a single dose of CRH 10^{-7} M. After CRH 10^{-7} M pretreatment, strips were rinsed an additional five times with Krebs buffer, to displace CRH bound to CRHRs, before the subsequent challenge with CRH 10^{-12} to 10^{-6} M. This method was modified from 1) the original CRHR desensitization studies performed in cultured cells in Grammatopoulos' laboratory, where cells were rinsed once with DMEM culture medium (27) and from 2) functional studies of homologous desensitization of GPCRs in other muscle types, where muscle strips were washed with Krebs buffer three times before a subsequent challenge with agonist (34). There was no difference in the maximal relaxatory response achieved by CRH alone (29 ± 9%) or in the presence of CRH pretreatment (45 ± 15%, $P > 0.05$, ANOVA), and no shift in the dose-response curves (n = 3 for each data set, data not shown). The potency of

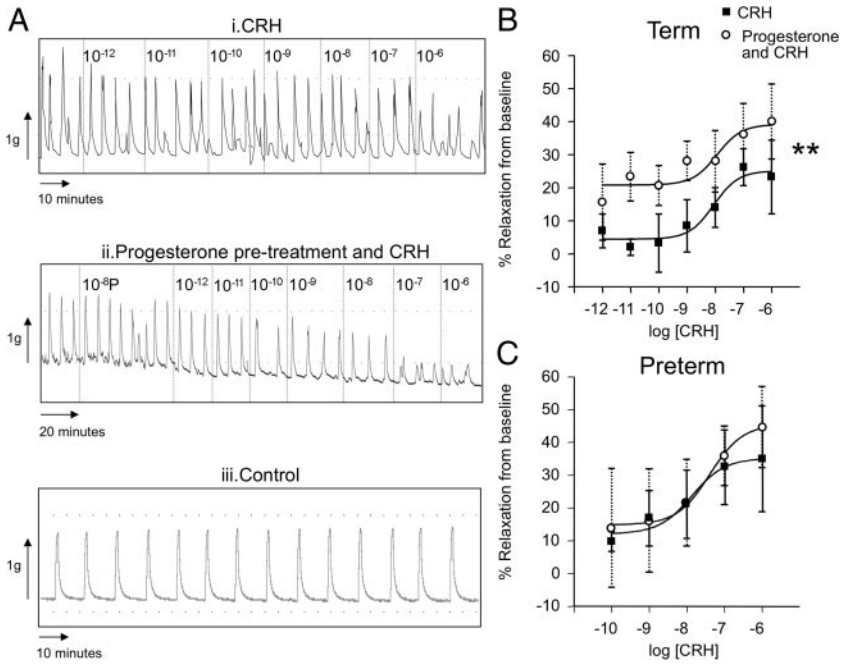

FIG. 3. CRH-induced myometrial relaxation in the presence and absence of P4. A, Representative recordings of spontaneous contractions in matched human myometrial strips after cumulative doses of CRH 10^{-12} to 10^{-6} M (i), P4 10^{-8} M as pretreatment (60 min) and then CRH 10^{-12} to 10^{-6} M (ii), or untreated control (iii). Cumulative effect of CRH on contractile activity, in the presence and absence of P4 in term (B) and preterm (C) myometria. Data points represent the mean ± SEM. **, $P < 0.01$ (ANOVA). Relaxation was measured as integrated area under the tension curve.

CRH (EC_{50} 1.1×10^{-9} M) was not altered by high-dose CRH pretreatment (EC_{50} 3.9×10^{-8} M).

Relaxatory response to salbutamol is unchanged in the presence of high-dose CRH

GPCRs may be subject to heterologous desensitization. To determine whether high concentrations of CRH cause heterologous desensitization of the β_2-R, the effect of cumulative doses of salbutamol was examined in the presence of CRH (30 min pretreatment with 10^{-9}, 10^{-8}, or 10^{-7} M CRH). In these experiments, salbutamol alone caused a maximal reduction in contractility of 43 ± 9% (mean ± SEM) (Fig. 5A). Salbutamol-induced relaxation was not altered by CRH pretreatment, with no significant shift of the dose-response curves ($P > 0.05$, ANOVA). The maximal effect and potency of salbutamol was unchanged in the presence of CRH (Fig. 5B). Treatment with a single dose of CRH (10^{-9}, 10^{-8}, or 10^{-7} M) exerted no significant change in contractility from baseline (data not shown).

Discussion

The precise function of CRH in the pregnant myometrium is unknown. CRH is postulated to exert a complex effect on contractility, being capable of inhibiting myometrial contractions as well as coordinating the transition from a quiescent state to an actively contracting tissue (35). We document for the first time CRH-induced relaxation of the lower uterine segment *in vitro* in both preterm and term strips of myometrium. In addition, treatment with P4 significantly enhanced the relaxatory effect of CRH in term myometria. PDE inhibition by rolipram potentiated both CRH- and salbutamol-induced relaxation, suggesting PDE activity contributes to heterologous down-regulation of cAMP/protein kinase A (PKA) signaling pathways in term myometria. Finally, the functional studies presented here do not support the hypothesis that high concentrations of CRH result in down-regulation of agonist-stimulated AC/cAMP transduction pathways, with consequent impaired agonist-mediated inhibition of contraction. No attenuation of relaxant response to CRH or salbutamol was observed in the presence of high-dose CRH.

Although several studies confirm the ability of CRH to increase myometrial cAMP (14, 15), it has been difficult *in vitro* to demonstrate CRH-induced inhibition of myometrial contractions in early studies using single doses of CRH (20). In our study, cumulative doses of CRH (10^{-12} to 10^{-6} M) caused a modest dose-dependent inhibition (31 ± 8%, n = 12) of contraction in term myometria obtained from the lower segment (Figs. 1 and 2A). Zhang *et al.* (22) using 10^{-10} to 10^{-7} M CRH also found an approximately 32 ± 5% reduction in activity integral in term NL samples. Mignot's group (21) examined CRH 10^{-10} to 10^{-6} M in samples from 36–40 wk gestation obtained from the uterine corpus (between the fundus and lower segment). Although CRHR1 mRNA is differentially expressed between the fundus and lower segment (12), the magnitude of the inhibitory response to cumulative doses of CRH in our study appears comparable to the approximately 30% reduction in area ratios under the contraction curve reported by Mignot *et al.* (21). Currently, no quantitative CRHR protein data are available for the different regions of the uterus or through the stages of gestation.

We hypothesized that CRH-induced relaxation would be greater in preterm myometria because CRH-stimulated cAMP production is diminished in term compared with preterm myometria (14). The difference reported in CRH-stimulated cAMP levels is modest, with about

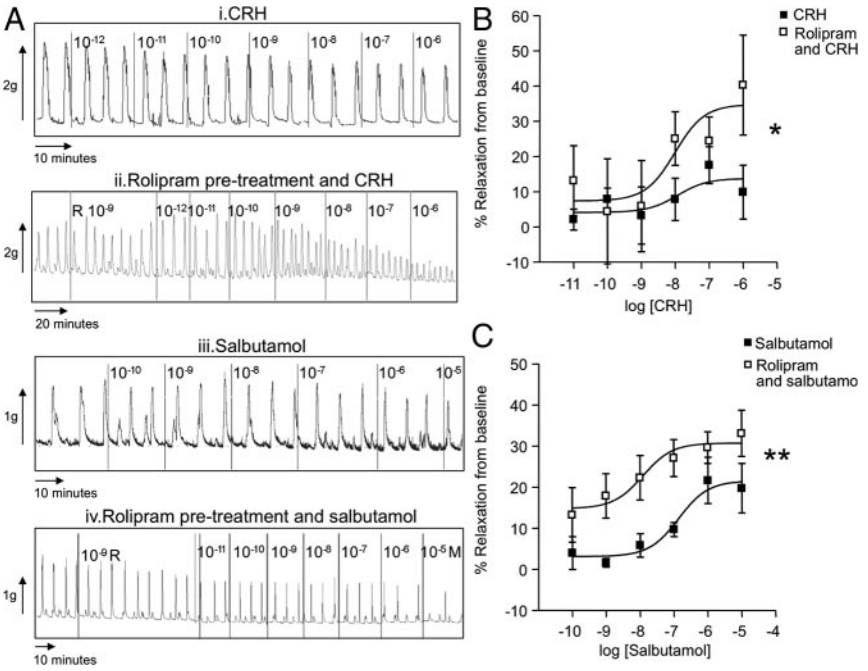

FIG. 4. CRH and salbutamol-induced myometrial relaxation in the presence and absence of rolipram. A, Representative recordings of spontaneous contractions in matched human myometrial strips after cumulative doses of CRH 10^{-12} to 10^{-6} M (i), rolipram10^{-9} M as pretreatment (60 min) and then CRH 10^{-12} to 10^{-6} M (ii), salbutamol 10^{-11} to 10^{-5} M (iii), or rolipram 10^{-9} M as pretreatment (60 min) and then salbutamol 10^{-11} to 10^{-5} M (iv) B, Cumulative effect of CRH on contractile activity in the presence and absence of rolipram. C, Cumulative effect of salbutamol on contractile activity in the presence and absence of rolipram. Data points represent the mean ± SEM. Relaxation was measured as integrated area under the tension curve. *, $P < 0.05$; **, $P < 0.01$ (ANOVA).

150% increase of basal cAMP levels in term and about 170% in preterm samples, after cumulative doses of CRH to 10^{-6} M. In the present study, we observed no functional difference in CRH-induced relaxation between term and preterm strips (Figs. 1 and 2A). The preterm group (n = 3) is small, due to the difficulty of obtaining NL preterm human samples, and a larger sample size might enable the identification of a modest difference. Although a relaxant effect was noted in the preterm samples, the bioavailability of circulating plasma CRH before 36 wk gestation is less clear, due to the higher levels of CRH-binding protein (4, 36). However, CRH is also produced locally in the myometrium and could exert additional paracrine or autocrine effects (37, 38).

No correlation was noted between gestational age and the effect of CRH in NL samples (Fig. 2B), with wide variability in sensitivity to CRH observed. Forskolin, a direct AC activator produced more consistent relaxation (Fig. 2D). The peptide hormone relaxin, an endogenous stimulant of myometrial cAMP implicated in uterine quiescence, similarly produces a mild and variable inhibitory effect on spontaneous contractions in term human myometria (39). The varied relaxant effect of CRH was also noted by Mignot's group (21). This is likely to reflect a complex interplay between myriad factors *in vivo*, including the balance between local concentrations of CRH and CRH-binding protein, dynamic changes in CRHR subtypes or $G\alpha_s$ with potential alteration in linkage to second messenger signaling pathways, and influence by other pathways such as oxytocin-stimulated signaling (26).

Given the variable sensitivity of the myometrium to CRH, smaller groups of paired samples were used to investigate effects of any pretreatment. We demonstrate for the first time significant enhancement of the relaxant effect of CRH in the presence of P4 or rolipram (Figs. 3 and 4). There was a 1.5-fold increase in maximal CRH-induced relaxation with P4, with significant displacement of the dose-response curves (Fig. 3B). Relaxation in term myometrial strips at 10^{-9} M CRH, reflective of *in vivo* concentrations in late gestation (10^{-9} to 10^{-8} M) (4) increased about 3.5-fold from 8 ± 8 to 28 ± 7% (mean ± SEM, n = 6) with concurrent P4 (Fig. 3B). The increased efficacy of CRH in the presence of P4 provides important functional evidence to support the hypothesis that CRH *in vivo* contributes to myometrial quiescence. This synergistic effect of P4, with increased inhibition of myometrial contractions *in vitro* has been described in conjunction with other agonists stimulating cAMP production, including relaxin (40) and the β_2-agonist ritodrine (41); the latter study similarly noted a vertical displacement of the ritodrine dose-response curves with P4, despite no overt relaxation produced by P4 10^{-8} M alone.

The exact mechanism whereby P4 augments cAMP-mediated relaxation is unknown, but the current experiments suggest a nongenomic effect. Early studies report a dose-dependent PDE inhibition induced by P4, observed in human pregnant myometrial tissue (32) and in cultured sheep myometrial cells (33). No enhancement of CRH with P4 was observed in the smaller preterm group (Fig. 3C); although PDE activity is well characterized in term myometria compared with the nonpregnant state, little information exists regarding PDE activity in preterm human myometria. Interestingly, prolonged P4 treatment of human myometrial cells reportedly alters transcription of the CRHR1 gene, with increased expression of the CRHR1α variant, which efficiently couples to $G\alpha_s$/AC/cAMP, compared with CRHR1β (42). It is possible that a

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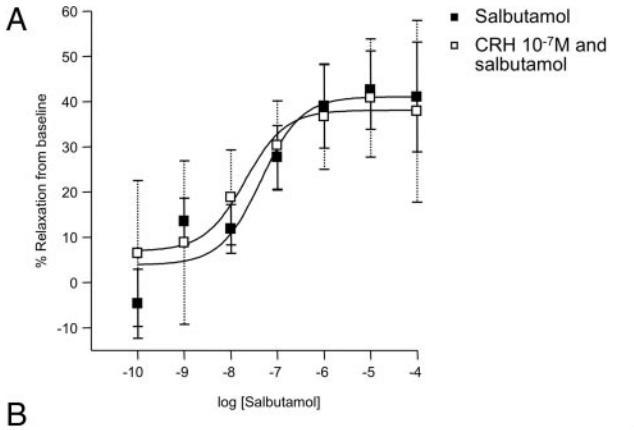

B

Treatment	Response		
	E_{max}±SEM	EC_{50}	95% CI EC_{50}
Salbutamol	43±9% (n=5-9)	4.4 x 10^{-8} M	2.4 x 10^{-9} to 7.9 x 10^{-7} M
CRH 10^{-9}M and Salbutamol	38±19% (n=3)	8.0 x 10^{-9} M	3.6 x 10^{-10} to 1.8 x 10^{-7} M
CRH 10^{-8}M and Salbutamol	29±4% (n=3-5)	3.4 x 10^{-10} M	5.5 x 10^{-12} to 2.1 x 10^{-8} M
CRH 10^{-7}M and Salbutamol	45±12% (n=4-8)	2.0 x 10^{-8} M	8.4 x 10^{-9} to 4.9 x 10^{-8} M

FIG. 5. Salbutamol-induced myometrial relaxation in the presence and absence of CRH. Myometrial strips were treated with salbutamol 10^{-10} to 10^{-4} M alone or in the presence of CRH (10^{-9} to 10^{-7} M, pretreatment for 30 min). A, Cumulative effect of salbutamol on contractile activity in the presence and absence of CRH 10^{-7} M. Data points represent the mean ± SEM. B, Effect of cumulative doses of salbutamol on spontaneous contractile activity in the presence and absence of CRH. E_{max} is the maximal observed effect in range of concentration tested. EC_{50} was calculated after fitting of idealized dose-response curves using GraphPad Prism software. CI, Confidence interval.

functional P4 withdrawal in late term may therefore lessen the relaxant effect of CRH.

P4 and rolipram enhanced the effect of CRH to a similar degree (Figs. 3B and 4B). Selective PDE4 inhibition significantly augmented the relaxant effect of not only CRH but also the β_2-agonist salbutamol in term myometria (Fig. 4). This confirms previous studies, where potentiation of salbutamol-induced relaxation with rolipram was noted (43, 44). As with the current study, Bardou *et al.* (44) reported a significant upward shift of the salbutamol dose-response curves in the presence of 3×10^{-8} M rolipram, suggesting an immediate enhancement of salbutamol-induced relaxation. It might be expected that the rational combination of known stimulants of cAMP with an inhibitor of cAMP degradation would result in an additive effect on relaxation; however, Méhats *et al.* (43) have also reported that selective PDE4 inhibition in nonpregnant myometrial strips failed to enhance salbutamol-mediated relaxation. Thus up-regulation of PDE4 at term (30, 31) is a likely mechanism for heterologous down-regulation of agonist-stimulated AC/cAMP/PKA pathways. In other tissues, intracellular cAMP levels regulate PDE expression; however, the factors regulating expression of myometrial PDE genes through gestation are not known (45). In human nonpregnant myometrial cells, long-term cAMP elevation caused a marked increase in PDE4 activity, with an accumulation of PDE4B and -4D variants (46). Treatment of pregnant women with the β_2-agonist terbutaline for preterm labor has also been associated with significant increases in myometrial PDE activity (47). It is plausible that the high concentrations of CRH characteristic of late pregnancy may similarly up-regulate PDE4 through chronic cAMP stimulation. CRH could therefore contribute to PDE-induced heterologous down-regulation of cAMP/PKA relaxatory pathways near labor.

The current studies also examined an alternative hypothesis, that CRH may induce down-regulation of cAMP/PKA relaxatory pathways at the level of GPCRs, through GRK recruitment. GRK 6 mediates homologous desensitization of both CRHR1α and the β_2-R in cultured cells (27, 28). Desensitization of these GPCRs leads to reduced agonist-stimulated cAMP production, which could result in impaired agonist-mediated relaxation. In a functional model to test the effect of CRH on β_2-agonist-mediated relaxation, high-dose CRH pretreatment did not alter salbutamol-induced relaxation (Fig. 5). Additionally, to determine whether homologous desensitization of CRHR results in attenuation of CRH-mediated relaxation, a single high dose of CRH (10^{-7} M) was followed by a second challenge with CRH (10^{-12} to 10^{-6} M). With CRH pretreatment, relaxation induced by subsequent doses of CRH was not impaired; thus we find no evidence for homologous down-regulation of CRHR-stimulated signaling pathways in our model. Cell-based studies report homologous desensitization of CRHR1 occurs in minutes, with subsequent recovery and full restoration of cAMP response within 2 h (25, 27). It is possible that the CRHR1α-stimulated cAMP response had largely recovered by the end of our protocol, because typical duration of the subsequent challenge to cumulative doses of CRH occurred over 2 h.

In conclusion, our studies provide important functional evidence confirming the ability of CRH to induce relaxation in term and preterm myometria. CRH at physiological concentrations acts synergistically with P4 to contribute to myometrial quiescence. The reported up-regulation of PDE4 activity in late pregnancy provides a mechanism for heterologous down-regulation of agonist-stimulated AC/cAMP/PKA pathways (31, 43). Collectively, these data lead to the formulation of a new hypothesis regarding CRH as a facilitator in the transition of the myometrium from relaxation to contraction. It is conceivable that high levels of CRH at term up-regulate PDE activity through

chronic cAMP elevation. CRH could therefore play an important role in PDE-mediated down-regulation of agonist-stimulated AC/cAMP/PKA pathways in late term, shifting the balance from a dominance of relaxatory pathways to favor the contractile phenotype required for successful labor.

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