The role of a cAMP-dependent pathway in the uterine relaxant action of relaxin in rats

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The aim of this study was to investigate the role of the adenylyl cyclase pathway, and in particular cyclic AMP-dependent protein kinase A, in the relaxant action of relaxin in the isolated uterus of the nonpregnant rat. The purportedly selective inhibitor of cAMP-dependent protein kinase A *N*-[2-(methylamino) ethyl]-5-isoquinolinesulfonamide hydrochloride (H-8) (at 100 $\mu\text{mol l}^{-1}$) antagonized relaxin, salbutamol (an agonist at β -adrenoceptors) and levcromakalim (a K^+ channel opener) to a similar extent (by factors of 3.1, 1.9 and 2.8, respectively), demonstrating that it is not a selective inhibitor. Relaxin and levcromakalim were less potent and had smaller, maximal, relaxant effects in longitudinal myometrium than in intact uterus cut in the longitudinal plane. By contrast, nifedipine (a Ca^{2+} channel blocker) was equipotent in the two preparations and salbutamol only slightly less potent in the longitudinal myometrium. Relaxin did not alter the cyclic AMP-dependent protein kinase A activity ratio in longitudinal myometrium but did increase the activity ratio by a factor of 2.0 ± 0.2 in the intact uterus. Salbutamol, the positive control, increased this activity ratio in both longitudinal myometrium (by 1.9 ± 0.3 times) and in the intact uterus (by 3.8 ± 0.3 times), whereas the negative control levcromakalim had no effect. Relaxin seems to act as a relaxant of longitudinal myometrium by a cyclic AMP-independent mechanism but possibly interacts with the circular myometrium or endometrium to release a relaxant factor via a cyclic-AMP-dependent mechanism.

Introduction

Relaxin, a polypeptide hormone, is a potent relaxant of uterine smooth muscle (Downing and Hollingsworth, 1993). However, the signal-transduction pathway underlying this effect is uncertain (Hughes and Hollingsworth, 1995, 1996). One hypothesis is that relaxin interacts with plasmalemma receptors in myometrial cells and activates adenylyl cyclase, resulting in the increased formation of cAMP and activation of cAMP-dependent protein kinase A (PKA) (Sanborn *et al.*, 1980, 1994).

Evidence supporting the existence of this pathway is the fact that relaxin increases cAMP concentrations in the uterus *in vitro* (Cheah and Sherwood, 1980; Judson *et al.*, 1980; Sanborn *et al.*, 1980; Hsu *et al.*, 1985; Osa *et al.*, 1991) and after treatment *in vivo* (Downing *et al.*, 1992). In addition, relaxin increases the total activity of protein kinase A (PKA) in the rat uterus (Kemp and Niall, 1981) and the inhibitory action of relaxin on the oxytocin-induced rise in the intracellular concentration of calcium, $[\text{Ca}^{2+}]_i$, is blocked by the purportedly selective PKA inhibitor *N*-[2-(methylamino) ethyl]-5-isoquinolinesulfonamide hydrochloride (H-8) (Hidaka *et al.*, 1984; Hagiwara *et al.*, 1987) in rat myometrial cells in culture

(Anwer *et al.*, 1990). H-8 also antagonizes relaxin as a uterine relaxant (Sanborn *et al.*, 1994).

There are, however, some results that do not support the role of this pathway in the inhibitory action of relaxin. First, relaxin stimulates an increase in uterine cAMP concentrations only when a phosphodiesterase inhibitor is present (Sanborn *et al.*, 1980; Hsu *et al.*, 1985). Second, the time courses for the increase in uterine cAMP concentrations *in vitro* (Sanborn *et al.*, 1980; Hsu *et al.*, 1985) and for PKA activation (Kemp and Niall, 1981) are slower than those for mechanical inhibition. Third, a prolonged inhibition of contractions by relaxin *in vivo* is associated with only a short duration of raised cAMP concentrations in the uterus *ex vivo*, and doses of relaxin and of salbutamol (an agonist at β -adrenoceptors that induces mechanical inhibition comparable with that of relaxin) induced markedly different increases of cAMP concentrations (relaxin increases it threefold while salbutamol increases it 19-fold) (Downing *et al.*, 1992). Finally, low concentrations of relaxin have a pharmacological profile consistent with a primary site of action at the plasmalemma in the modification of ion channel activity (Hughes and Hollingsworth, 1995). Similar inconsistencies have been identified for the cardiac stimulant actions of relaxin. Summers *et al.* (1994) failed to observe any changes in cAMP concentrations in the rat atria in response to relaxin, whereas Han *et al.* (1994) found that a protein kinase inhibitor prevented the stimulant effects of relaxin in rabbit hearts.

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Such inconsistencies may be partially explained if the changes in cAMP concentrations in any tissue are too small to be readily detected, and hence do not reflect the activation of enzymes further down the cascade of intracellular events and the end physiological effect (Corbin, 1983). Small changes in cAMP are often associated with larger changes in PKA activity (Corbin, 1983), a feature known as amplification (Kenakin, 1993), and so PKA activity may be a preferable biochemical index. PKA is present in the rat uterus, both in the endometrium and myometrium (Korenman and Krall, 1977). cAMP–PKA consists of two regulatory and two catalytic subunits (Corbin, 1983). When cAMP binds to a regulatory subunit, an active catalytic subunit is released from the holoenzyme. Hence, PKA activity is commonly given as the PKA activity ratio, which is the amount of PKA in the active form expressed as a fraction of the total PKA in the tissue in the presence of excess added cAMP (Corbin, 1983).

Another explanation for some of the inconsistencies listed above may be that relaxin acts partly or totally indirectly via the release of a factor from the endometrium. Although some of the biochemical studies have been conducted with myometrial cells (Anwer *et al.*, 1990; Sanborn *et al.*, 1994), many have been carried out on the whole uterus and so may reflect in part changes in the endometrium and not the myometrium. A potential mediator is nitric oxide, which is synthesized in the rat uterus and is a relaxant of myometrium (Yallampalli *et al.*, 1993).

The aim of this study was, therefore, to investigate the role of the adenylyl cyclase pathway in the mechanism of the relaxant action of relaxin in the isolated uterus of nonpregnant rats, and to determine whether the action was direct or indirect. These aims were achieved by determining (1) the ability of the PKA inhibitor H-8 or N^ω nitro L-arginine methyl ester (L-NAME), an inhibitor of nitric oxide synthase (Norman, 1996), to antagonize relaxin in tissue bath experiments, and (2) the ability of relaxin to increase the PKA activity ratio both in longitudinal myometrium and in the intact uterus (longitudinal plus circular myometrium and endometrium). The action of relaxin was compared with that of three standard relaxants: levromakalim, a K⁺-channel opener the actions of which do not involve the adenylyl cyclase pathway (Piper *et al.*, 1990; Edwards and Weston, 1994); salbutamol, a β -adrenoceptor agonist, for which there is much evidence supporting its role in the adenylyl cyclase pathway (Diamond, 1990); and nifedipine, a Ca²⁺-channel blocker (Granger *et al.*, 1986).

Materials and Methods

Animals

Female, nonpregnant Sprague–Dawley rats (200–300 g) were purchased from Charles River Ltd (Margate) and housed in the Biological Services Unit at Manchester University. Rats were pretreated with oestradiol benzoate (100 μ g kg⁻¹ in arachis oil, 1 ml kg⁻¹ s.c.) 18–24 h before experimentation. Rats were killed and uterine horns removed and placed in a physiological salt solution (PSS).

Drugs and solutions

Levcromakalim (SmithKline Beecham, Welwyn Garden City), salbutamol sulfate (Glaxo, Stevenage), nifedipine (Bayer, Newbury), oxytocin acetate, oestradiol benzoate, EDTA, Tris–HCl, 3-isobutyl-1-methylxanthine (IBMX), kemptide, cAMP, protein kinase inhibitor (PKI) (6–22)amide (Thr6-Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile-22-NH₂), L-NAME, BSA (Sigma, Poole), H-8 (Research Biochemicals Incorporated, St Albans), concentrated phosphoric acid (Aldrich, Gillingham), [³²P]ATP (167 TBq mmol l⁻¹; ICN Biomedical Inc., Thame), and Ecoscint (National Diagnostics, Wigan) were used during the course of the experiments.

Porcine relaxin was isolated from pregnant sow ovaries according to the method of Sherwood and O'Byrne (1974) by S. J. Downing (Department of Biology, University of York). Relaxin was bioassayed *in vitro* by inhibiting the electrically stimulated uterus from rats that had been treated with 100 μ g oestrogen kg⁻¹; porcine relaxin, kindly donated by O. D. Sherwood (Department of Physiology and Biophysics, University of Illinois), was the standard. The relaxin preparation was found to be equipotent with highly purified relaxin.

Stock solutions were prepared in the following solvents: levromakalim in 70% (v/v) ethanol; salbutamol in 0.1 mol HCl l⁻¹; relaxin in saline; oxytocin acetate, nifedipine and H-8 in double-distilled water; oestradiol benzoate in arachis oil. Serial dilutions of stock solutions were made in saline on the day of use. The PSS had the following composition: 143.0 mmol Na⁺ l⁻¹, 5.9 mmol K⁺ l⁻¹, 1.2 mmol Mg²⁺ l⁻¹, 2.6 mmol Ca²⁺ l⁻¹, 1.2 mmol H₂PO₄⁻ l⁻¹, 1.2 mmol SO₄²⁻ l⁻¹, 128.0 mmol Cl l⁻¹, 25.0 mmol HCO₃⁻ l⁻¹, and 11.0 mmol glucose l⁻¹.

Tissue bath experiments

Tissue preparation. Experiments investigating the effects of H-8 or L-NAME upon the three relaxants were performed upon tubular segments of intact uterus that were prepared for the recording of tension in the longitudinal plane. For experiments comparing the effects of the relaxants on tension development in the intact uterus and separated longitudinal myometrium, the two tubular horns of each uterus were cut along the mesometrial border, opened out and pinned to a cork board. The tissue of one horn was left intact while the endometrium and circular muscle layer were removed from the other horn using a pair of fine forceps. The tissue from each horn was cut into two equal rectangular segments and prepared for the isometric recording of tension. Tissues were mounted in 10 ml tissue baths containing PSS at 37°C, gassed with 95% O₂ and 5% CO₂, and allowed to equilibrate under 1 g tension for 30–60 min before experimentation. Mechanical responses were measured as the integral of the tension versus the time curve, as described by Granger *et al.* (1985).

Antagonism of the relaxants by H-8 or by L-NAME. After equilibration in PSS, the tissues were incubated in 100 μ mol H-8 l⁻¹ (as described by Anwer *et al.*, 1990) (test tissues) or vehicle (control tissues) for 20 min before adding PSS containing 0.2 nmol oxytocin l⁻¹ plus H-8 or vehicle. After equilibration for 15 min with 0.2 nmol oxytocin l⁻¹, a single,

cumulative, concentration–effect curve was constructed for relaxin, levcromakalim or salbutamol in the continued presence of H-8 or vehicle. The concentration of relaxin, levcromakalim or salbutamol was doubled every 10 min until 100% or maximal inhibition was achieved. The same experimental design was used with 10 $\mu\text{mol L-NAME l}^{-1}$.

Effects of relaxants on oxytocin-induced phasic spasm in intact uterus and longitudinal myometrium. After equilibration in PSS, the tissues were incubated for 15 min in PSS containing 0.2 nmol oxytocin l^{-1} . One of the two segments of each intact uterus and of longitudinal myometrium was exposed to oxytocin-containing PSS but not to relaxants, to measure any decline of the spasm with time. In the other segment, a single, cumulative, concentration–effect curve was constructed for relaxin, salbutamol, nifedipine or levcromakalim.

Statistical analysis. The integrated tension recorded during the 5–10 min period after the addition of each concentration of drug is expressed as a percentage of the integrated tension recorded during the 5 min period before drug addition; this gives a value for the percentage spasm remaining in the presence of relaxant. pD_2 values and $\log_{10}$ concentration ratios were calculated and statistical analysis was performed as described by Hughes and Hollingsworth (1995).

Determination of the PKA activity ratio and the effects of relaxants

PKA activity was monitored by measuring the incorporation of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ into a substrate protein kemptide, which was monitored by liquid scintillation counting. The tissue extracts were assayed under four conditions: the absence and presence of cAMP and of PKI(6–22)amide, the purported selective inhibitor of cAMP-dependent PKA (Glass *et al.*, 1989). This approach ensured that activity specific to this PKA was monitored.

Preparation of tissue homogenate. On each experimental day, both horns of each uterus were prepared in the same manner to give four tissue segments of either intact uterus or longitudinal myometrium. The tissues were first mounted in tissue baths to record tension changes, as described above. Tissues were exposed to PSS containing 0.2 nmol oxytocin l^{-1} for 15 min before exposure to a single concentration of relaxant (salbutamol, relaxin or levcromakalim) for 1 min. In one experiment, each tissue was exposed to the same concentration of relaxin (300 nmol l^{-1}) but for various durations (1, 2, 4 or 6 min). After incubation with relaxant, the tissues were removed from the tissue baths and frozen in liquid nitrogen. Each frozen tissue was powdered and transferred to a glass test tube containing 1 ml extraction buffer (50 mmol Tris–HCl l^{-1} , 10 mmol EDTA l^{-1}), 0.5 mmol IBMX l^{-1} and 100 mmol sodium chloride l^{-1} and was stored on ice while the rest of the tissues were prepared. The tissue homogenates were centrifuged in a Mistral 6000 centrifuge at 4°C for 5 min at 6000 r.p.m. The tubes containing the pellet and the supernatant were stored on ice until required.

Assay procedure. The procedure was modified from that of the Gibco BRL (Uxbridge) Protein Kinase A Assay System and methods used by Corbin *et al.* (1983) and Murray *et al.* (1990). The assay was performed in Eppendorf tubes in duplicate. The tissue supernatants were incubated in substrate buffer (50 mmol Tris–HCl l^{-1} , 40 mmol $\text{MgCl}_2 \text{l}^{-1}$, 400 $\mu\text{mol ATP l}^{-1}$, 1 mg BSA ml^{-1} and 200 $\mu\text{mol kemptide l}^{-1}$) with or without cAMP and/or PKI(6–22)amide at room temperature for 15 min. This approach allowed the cAMP to activate the enzyme and/or PKI(6–22)amide to bind to any PKA and ensured that activity specific to cAMP-dependent PKA was measured. The tubes were transferred to an Eppendorf mixer, maintained at 30°C, and 20 μl of $[\text{}^{32}\text{P}]\text{ATP}$ /substrate solution was added to each tube to initiate the reaction. The assay tubes each contained the following final concentrations: 50 mmol Tris–HCl l^{-1} (pH 7.5), 10 mmol $\text{MgCl}_2 \text{l}^{-1}$, 100 $\mu\text{mol } [\text{}^{32}\text{P}]\text{ATP l}^{-1}$ (8 nmol per assay tube; 167 TBq per mmol), 0.25 mg BSA ml^{-1} , and 50 $\mu\text{mol kemptide l}^{-1}$, with or without 10 $\mu\text{mol cAMP l}^{-1}$ or 1 $\mu\text{mol PKI(6–22)amide l}^{-1}$. The assay tubes were incubated for 6 min. The reaction was terminated by spotting 40 μl of the 80 μl of solution on to phosphocellulose paper (Whatman, Maidstone), which was washed twice with 250 ml phosphoric acid (1%, v/v) and a further three times with water to remove $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. The squares of phosphocellulose were put into scintillation vials and 3 ml Ecoscint scintillant was added. The radioactive ^{32}P label was determined by liquid scintillation counting (Packard Tricarb 460C Liquid Scintillation Counter). A 20 μl sample of the $[\text{}^{32}\text{P}]\text{ATP}$ /substrate solution was counted to measure the total activity added to the reaction mixture and a square of phosphocellulose, washed only with phosphoric acid and water, was counted to measure the background count.

Statistical analysis. The rationale is as described in the Gibco BRL kit. The PKA activity ($\text{nmol min}^{-1} \text{ml}^{-1}$) was calculated for each of the four conditions. The amount of activated PKA due to relaxant expressed as a proportion of the total PKA activity gave the decimal fraction of activated PKA, or PKA activity ratio. Mean activity ratios for control and drug-exposed groups were compared using the Kruskal–Wallis non-parametric test of analysis of variance, followed by Mann–Whitney analysis between pairs to locate differences; $P < 0.05$ was taken as significant.

Results

Relaxant inhibition of oxytocin-induced spasm and the effects of H-8 and L-NAME

These experiments were performed in the intact uterus. Oxytocin induced phasic spasms that were regular in frequency and amplitude, and the integral of spasm was well maintained for the duration of the experiment (Hughes and Hollingsworth, 1995). Cumulative addition of levcromakalim (0.025–0.8 $\mu\text{mol l}^{-1}$), salbutamol (0.125–8 $\mu\text{mol l}^{-1}$) or relaxin (0.8–50 nmol l^{-1}) produced a reversible, concentration-dependent reduction of the integral of oxytocin-induced tension waves (Fig. 1). H-8 (100 $\mu\text{mol l}^{-1}$) shifted the $\log_{10}$ concentration–effect curves for salbutamol and relaxin to the right by factors of 1.9 and 3.1, respectively (Fig. 1; Table 1). In the presence of H-8

Fig. 1. The effect of *N*-[2-(methylamino)ethyl]-5-isoquinoline-sulfonamide hydrochloride (H-8; 100 μmol l⁻¹) on the inhibition of spasm (induced by oxytocin; 0.2 nmol l⁻¹) evoked by (a) levromakalim, (b) salbutamol, and (c) relaxin in intact, isolated uteri from nonpregnant rats pretreated with oestradiol. Effects are shown in the absence (○) and presence (●) of H-8. The y axes show the spasm remaining as a percentage of that recorded before any relaxant was added. The x axes are the log₁₀ concentrations of relaxant. Points represent the means and the vertical lines the SEM of six–eight tissues.

(100 μmol l⁻¹), the log₁₀ concentration–effect curve for levromakalim was shifted to the right by a factor of 2.8. The maximal effects of levromakalim and relaxin were reduced to only 60–70% by H-8. There was no difference in the extent of

antagonism of each of the three relaxants by H-8 at 100 μmol l⁻¹ ($P > 0.05$). L-NAME (10 μmol l⁻¹) did not antagonize relaxin (pD_2 in the absence of L-NAME = 7.87 ± 0.6 , $n = 4$; pD_2 in the presence of L-NAME = 8.05 ± 0.26 , $n = 4$; $P > 0.05$).

Effects of relaxants on oxytocin-induced spasm in intact uterus and longitudinal myometrium

Salbutamol (0.25–32 nmol l⁻¹) and nifedipine (1–128 nmol l⁻¹) completely inhibited the spasm evoked by oxytocin (0.2 nmol l⁻¹) in both tissue preparations. There was a small reduction (by a factor of 2.6; $P < 0.05$) in the potency of salbutamol against oxytocin-induced spasm in the longitudinal myometrium ($pD_2 = 8.71 \pm 0.11$, $n = 6$) compared with that measured in the intact uterus ($pD_2 = 9.12 \pm 0.09$, $n = 8$; Fig. 2). Nifedipine was equipotent against the oxytocin-induced spasm in the intact uterus ($pD_2 = 7.93 \pm 0.06$, $n = 4$) and longitudinal myometrium ($pD_2 = 8.01 \pm 0.08$, $n = 4$; Fig. 2). Levromakalim completely inhibited the oxytocin-induced spasm in the intact uterus with a pD_2 of 6.91 ± 0.08 ($n = 8$) (Fig. 2). However, despite the addition of very high concentrations (50 μmol l⁻¹), levromakalim caused only a $61 \pm 7\%$ ($n = 13$) inhibition in the longitudinal myometrium (Fig. 2). Complete inhibition of the oxytocin-induced spasm was achieved by relaxin in the intact uterus ($pD_2 = 7.91 \pm 0.16$, $n = 8$) while in the longitudinal myometrium the maximum effect of relaxin was only a $58 \pm 5\%$ ($n = 7$) inhibition at a concentration of 2.5 μmol l⁻¹ (Fig. 2).

Characterization of the protein kinase A assay conditions

Preliminary experiments were performed to characterize the effects of dilution upon the reaction equilibrium and the inhibitory actions of PKI(6–22)amide on PKA activity before investigating drug actions on PKA activity.

The phosphorylation of the peptide substrate kemptide was linear with respect to the dilution of the tissue extract between dilutions of 1:4 and 1:16, and the activity ratio was unchanged for dilutions between 1:4 and 1:16 in both the control and test tissues. The PKA activity stimulated by excess cAMP (10 μmol l⁻¹) was almost completely inhibited by 1 μmol PKI(6–22)amide l⁻¹ ($97.7 \pm 0.5\%$, $n = 6$, for a 1:4 dilution). Hence, a dilution of 1:4 was used in the rest of this study.

Protein kinase A assay and the effects of relaxants

Salbutamol. The basal PKA activity ratio was slightly but significantly higher ($P < 0.05$) in tissue extracts from longitudinal myometrium than in extracts from the intact uterus (0.17 ± 0.02 and 0.12 ± 0.01 , respectively) (Fig. 3). The lowest concentration of salbutamol tested (30 nmol l⁻¹) did not modify the PKA activity ratio in either tissue preparation. The PKA activity ratio was raised following incubation with the higher concentrations of salbutamol (300 nmol l⁻¹, 3000 nmol l⁻¹) in both the intact uterus and longitudinal myometrium (Fig. 3a,b). In the presence of the highest concentration of salbutamol (3000 nmol l⁻¹), the PKA activity ratio in the intact

Table 1. The effect of H-8 ($100 \mu\text{mol l}^{-1}$) on spasm induced by oxytocin (0.2 nmol l^{-1}) in intact, isolated uteri from nonpregnant rats pretreated with oestradiol benzoate (control data are from tissues exposed to the vehicle for H-8 and test data are from tissues exposed to H-8)

	Control curve pD_2	Test curve pD_2	$\log_{10}$ concentration ratio	Concentration ratio
Levcromakalim	6.99 ± 0.06	6.54 ± 0.18	$0.45 \pm 0.19^*$	2.8
Salbutamol	8.97 ± 0.12	8.68 ± 0.08	$0.29 \pm 0.14^*$	1.9
Relaxin	8.63 ± 0.07	8.14 ± 0.12	$0.49 \pm 0.14^{**}$	3.1

Data presented are mean $\pm$ SEM values from six–eight tissues.

*($P < 0.05$) and **($P < 0.01$) indicate that the $\log_{10}$ concentration ratio was significantly different from 0.

H-8, N-[2-(methylamino)ethyl]-5-isoquinolinesulphonamide hydrochloride.

Fig. 2. Effect of (a) salbutamol, (b) nifedipine, (c) levcromakalim, and (d) relaxin on the spasm evoked by oxytocin (0.2 nmol l^{-1}) in intact uterus ($\bullet$) and longitudinal myometrium ($\circ$) from nonpregnant rats pretreated with oestradiol. The y axes show the spasm remaining as a percentage of that recorded before any relaxant was added. The x axes are the $\log_{10}$ concentrations of relaxant. Points represent the means and the vertical lines the SEM of 4–13 tissues.

uterus was 3.8 ± 0.3 times the basal activity ratio, whereas the PKA activity ratio in the longitudinal myometrium was 1.9 ± 0.3 times the basal activity ratio.

Relaxin. The basal PKA activity ratio was again slightly but significantly higher ($P < 0.05$) in tissue extracts from longitudinal myometrium than in extracts from intact uterus

(0.16 ± 0.03 and 0.11 ± 0.02 , respectively) (Fig. 4). Relaxin did not increase the PKA activity ratio in the longitudinal myometrium (Fig. 4a). However, in the intact uterus, relaxin induced a small and significant ($P < 0.05$) rise in the PKA activity ratio at concentrations of 300 nmol l^{-1} (1.8 ± 0.2 times the basal activity ratio) and 3000 nmol l^{-1} (2.0 ± 0.2 times the basal activity ratio; Fig. 4b).

Fig. 3. The effect of salbutamol on the cAMP-dependent protein kinase A activity ratio in tissue extracts from (a) longitudinal myometrium, and (b) intact uterus from nonpregnant rats pretreated with oestradiol. Bars represent the mean values and the vertical lines the SEM of four–five tissues. *($P < 0.05$) indicates a value that is significantly different from the control value.

Fig. 4. The effect of relaxin on the cAMP-dependent protein kinase A activity ratio in tissue extracts from (a) longitudinal myometrium, and (b) intact uterus from nonpregnant rats pretreated with oestradiol. Bars represent the mean values and the vertical lines the SEM of five tissues. *($P < 0.05$) indicates a value that is significantly different from the control value.

The time course of the effect of relaxin (300 nmol l⁻¹) upon PKA activity in the intact uterus is shown (Fig. 5). The PKA activity ratio was maximally raised (to 1.8 ± 0.2 times the control value) 1 min after the tissue had been exposed to relaxin, and remained raised for 6 min.

Levcromakalim. The basal value of the PKA activity ratio was measured in the intact uterus in the presence of the vehicle for levcromakalim; the concentration of the vehicle used corresponded to that applied when the highest concentration of levcromakalim (100 $\mu\text{mol l}^{-1}$) was used (0.28% (v/v) ethanol). This basal PKA activity ratio (0.12 ± 0.01) was not different ($P > 0.05$) from those values measured in the intact uterus on other experimental days. Levcromakalim (1–100 $\mu\text{mol l}^{-1}$) did not alter the PKA activity ratio in tissue extracts prepared from segments of intact uterus (PKA activity ratio for control, 0.12 ± 0.01 ; activity ratio for 1 $\mu\text{mol l}^{-1}$ levcromakalim, 0.12 ± 0.01 ; for 10 $\mu\text{mol l}^{-1}$, 0.12 ± 0.01 ; and for 100 $\mu\text{mol l}^{-1}$, 0.13 ± 0.01). The effect of levcromakalim on the PKA activity ratio in longitudinal myometrium was not assessed.

Discussion

The observation that relaxin is antagonized by the purportedly selective PKA inhibitor H-8 (at 100 $\mu\text{mol l}^{-1}$) in isolated myometrial cells (Hidaka *et al.*, 1984; Hagiwara *et al.*, 1987) led Anwer *et al.* (1990) to conclude that PKA has a role in the inhibitory action of relaxin against oxytocin-induced increases in inositol trisphosphate formation and intracellular Ca^{2+} ion concentrations. In the current study, salbutamol, a drug known to activate the adenylyl cyclase pathway, was antagonized by 100 $\mu\text{mol l}^{-1}$ H-8, the same concentration used by Anwer *et al.* (1990) and Sanborn *et al.* (1994). However, the degree of antagonism was small, which perhaps reflects the lack of intracellular penetration of H-8.

Levcromakalim was also antagonized by H-8 and to a similar extent to salbutamol. There is substantial evidence suggesting that the opening of a K^{+} channel is the primary mechanism of action of levcromakalim, with no involvement of adenylyl cyclase activation (Edwards and Weston, 1994). The simplest explanation for these observations is that H-8 is not a selective inhibitor of PKA in whole tissues. Recent data suggest that H-8 can inhibit protein kinases other than cAMP-dependent

Fig. 5. Time course of the action of relaxin (300 nmol l^{-1}) on the cAMP-dependent protein kinase A activity ratio in tissue extracts of the intact uterus from nonpregnant rats pretreated with oestradiol. Tissues were exposed to relaxin in the tissue bath for 1–6 min before the tissue extract was prepared. Bars represent the mean values and the vertical lines the SEM of four–five tissues. * ($P < 0.05$) indicates a value that is significantly different from the control value.

protein kinases (Fujii *et al.*, 1995); therefore, the effects observed could be due to inhibition of these kinases in addition to PKA.

Relaxin was antagonized by H-8 and to a similar extent to the two comparison drugs. However, in light of the antagonism of levromakalim, the antagonism of relaxin by H-8 cannot be taken to indicate that relaxin acts via PKA.

Control experiments demonstrated that the PKA assay could measure PKA activity in the uterus. The activity ratio was independent of dilution over the range used, showing that the holoenzyme did not dissociate during the procedure (Corbin, 1983; Murray *et al.*, 1990). The inhibitor PKI(6–22)amide ($1 \mu\text{mol l}^{-1}$) almost completely inhibited drug-induced activity, demonstrating activation and specific measurement of PKA.

It is well known that salbutamol activates the adenylyl cyclase pathway (Diamond, 1990); in this study, it acted as the positive control. In tissue bath studies, salbutamol was slightly less potent as a relaxant in the longitudinal myometrium than in the intact uterus. This observation might indicate that part of the inhibitory action of salbutamol in the intact uterus is mediated via the circular muscle layer or the endometrium. Alternatively, its reduced potency in the longitudinal myometrium may have resulted from damage inflicted to this layer when removing the circular muscle layer and the endometrium. However, the potency of nifedipine was the same in the two preparations, indicating that non-specific tissue damage probably did not occur and does not account for the reduced potency of salbutamol in the longitudinal myometrium. The PKA activity ratio was markedly increased by salbutamol in both the longitudinal myometrium and the intact uterus. Salbutamol induced a proportionally larger change in the PKA activity in the intact uterus than in the longitudinal myometrium, possibly reflecting the greater potency of salbutamol as a relaxant in the uterus.

Levromakalim induced complete inhibition of the oxytocin-induced spasm in intact uterus; however, the maximum effect

was only 60% inhibition in the longitudinal myometrium and this result could be achieved only in the presence of high concentrations of levromakalim. The maximum effect was similarly reduced in the presence of H-8. These observations suggest that the inhibitory action of levromakalim on the intact uterus is partly indirect and dependent upon a factor released from the circular muscle layer or the endometrium. Such observations have been observed in the rat uterus using cromakalim (Piper, 1992). Levromakalim did not stimulate the PKA activity ratio in the intact uterus. This negative result was expected since it is well known that the major action of levromakalim is to open K^+ channels (Edwards and Weston, 1994); it corresponds to observations made in other tissues in which cromakalim did not increase cAMP concentrations (Edwards *et al.*, 1992; Small *et al.*, 1992), and it serves to show that the PKA assay method is selective.

Relaxin produced partial inhibition of oxytocin-induced spasm in the longitudinal myometrium but did not stimulate the PKA activity ratio. Thus, the inhibitory action of relaxin in the longitudinal myometrium is unlikely to be mediated by activation of the adenylyl cyclase pathway. It was observed that salbutamol could produce relaxation in longitudinal myometrium at concentrations below those that increase PKA activity. However, relaxin, in the same ratio of biochemical to tissue bath concentrations as salbutamol, failed to increase the PKA activity ratio. Many previous reports of relaxin-induced increases in cAMP concentrations have not localized the changes to a specific layer of the uterus. Relaxin-induced increases in cAMP concentrations have been measured in cultured myometrial cells where tissue was taken from both muscle layers, longitudinal and circular (Hsu *et al.*, 1985), or changes were measured in intact uterus (Judson *et al.*, 1980; Grazi *et al.*, 1988).

Given the absence of any PKA stimulation in the longitudinal myometrium with relaxin, the stimulation of PKA seen in the intact uterus must be localized to the circular muscle layer or the endometrium. Unfortunately, it is technically extremely difficult to separate the circular myometrium from the endometrium. The observation that oxytocin-induced spasm was maximally inhibited by relaxin by only 60% in the longitudinal myometrium but that complete inhibition of this spasm was induced by relaxin in the intact uterus indicates that a part of the inhibitory action of relaxin in the intact uterus is effected via the circular muscle layer or the endometrium. It is possible that relaxin stimulates a cAMP–PKA-dependent process in the circular myometrium/endometrium that results in the release of a factor that acts in turn upon the longitudinal myometrium and leads to relaxation. It might have been speculated that the inhibitory factor is nitric oxide, which has been shown to be a relaxant of the uterus in the pregnant rat (Yallampalli *et al.*, 1993). However, relaxin was not antagonized by L-NAME, the nitric oxide synthase inhibitor, discounting NO as the intermediary.

In summary, the relaxant action of relaxin in the longitudinal myometrium is independent of cAMP-dependent protein kinase and presumably does not involve the adenylyl cyclase pathway. In contrast, relaxin has an action involving the adenylyl cyclase pathway in the circular myometrium and/or the endometrium and perhaps the release of a relaxant factor.

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