Cyclic adenosine 3'5'-monophosphate and the relaxant action of relaxin in the rat uterus *in vivo*

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Summary. The relationship between relaxant actions of relaxin in the uterus and changes in uterine cAMP concentrations was assessed in anaesthetized bilaterally ovariectomized nonpregnant rats. Relaxin i.v. bolus ($5 \mu\text{g kg}^{-1}$) did not change cAMP concentrations but inhibited uterine contractions with rapid onset. Uterine contractions were significantly reduced by 30–70% for 60 min. Relaxin ($50 \mu\text{g kg}^{-1}$) produced a short-lived (up to 5 min) and small (up to 3.2-fold) increase in cAMP concentrations plus a marked (90%) and prolonged inhibition of uterine contractions (70–90% over 60 min). Salbutamol (an agonist at β_2 -adrenoceptors, 100 and $500 \mu\text{g kg}^{-1}$) produced a similar degree and time course of inhibition of uterine contractions to that of relaxin but a more marked (19-fold) increase in cAMP concentrations. Glibenclamide, a blocker of ATP-sensitive potassium channels, which has been shown to antagonize relaxin as a uterine relaxant, did not prevent the relaxin-induced rise in cAMP concentrations. It is suggested that the uterine relaxant action of relaxin may not result from an increase in uterine cAMP concentrations.

Keywords: cAMP; relaxin; uterus; salbutamol; signal transduction; rat

Introduction

Relaxation of the uterus is an important physiological action of the hormone relaxin (Downing & Sherwood, 1985; Sherwood, 1988). This action of relaxin is seen within seconds of injection of relaxin *in vivo*, or administration of relaxin to isolated uterine tissue or to myometrial cells in culture and is usually reversible within minutes of its removal (Sanborn *et al.*, 1980; Bradshaw *et al.*, 1981; Hsu *et al.*, 1985). It is, therefore, likely that this action involves interaction of relaxin with a plasma membrane receptor and initiation of a signal transduction mechanism that results in enhanced enzyme activity, a change in the state of an ion channel or both actions.

Cyclic adenosine 3'5'-monophosphate (cAMP) has been suggested as the biochemical link between relaxin–receptor interaction and uterine relaxation (Sanborn & Anwer, 1990). Relaxin administration to isolated uterus or myometrial cells in culture produces a time- and concentration-dependent rise in cAMP concentrations (Cheah & Sherwood, 1980; Judson *et al.*, 1980; Sanborn *et al.*, 1980; Hsu *et al.*, 1985; Grazi *et al.*, 1988; Kramer *et al.*, 1990). In addition, relaxin inhibits the activity of myosin light chain kinase, decreases the extent of phosphorylation of myosin light chains and decreases myosin ATPase activity, all effects that may result from a rise in intracellular cAMP concentrations (Nishikori *et al.*, 1982, 1983). However, the changes in cAMP concentrations tend to follow rather than precede the mechanical inhibition. In addition, the rises in cAMP concentrations are usually seen only in the presence of a phosphodiesterase inhibitor, unlike agonists at β_2 -adrenoceptors the relaxant actions of which are paralleled by the accumulation of cAMP in the absence of such inhibitors (Hsu *et al.*, 1985).

A possible alternative mechanism by which relaxin produces uterine relaxation is via the opening of ATP-sensitive potassium (K^+) channels in the plasmalemma. Support for this

hypothesis comes from the observation that relaxin is antagonized by glibenclamide, a blocker of ATP-sensitive K^+ channels, in the uterus of the rat *in vivo* (Downing & Hollingsworth, 1991).

To date, biochemical changes have been measured only in the uterus after relaxin administration to the isolated tissue. The aim of the present study was to test further the hypothesis linking increased cAMP concentrations and relaxin-induced uterine relaxation. Cyclic AMP concentrations were measured *ex vivo* after porcine relaxin or β_2 -adrenoceptor agonist (salbutamol) administration in parallel with measurement of uterine relaxant actions in the rat *in vivo*. Relaxin-induced changes in cAMP concentrations were also measured after glibenclamide treatment and in relaxin-tolerant rats in which the uterus is less sensitive to the relaxant actions of relaxin (Downing & Hollingsworth, 1989, 1991).

Materials and Methods

Surgical procedures and experimental design for cAMP concentrations

Nonpregnant rats (170–340 g), at undefined stages of the oestrous cycle, were anaesthetized with tribromoethanol (240 mg kg^{-1} , i.p.; Fluka Chemicals, Glossop, Derbyshire, UK), bilaterally ovariectomized and equipped with a jugular vein cannula. All rats were given morphine sulfate (5 mg kg^{-1} ; Evans, Dunstable, Beds, UK) for post-operative analgesia. No steroid hormone treatment was given to rats before determination of uterine cAMP concentrations.

The first series of experiments assessed the acute effects of relaxin and salbutamol on uterine cAMP concentrations. Rats were re-anaesthetized with tribromoethanol i.v. 24 h after initial surgery and given a bolus dose of vehicle (saline, 1 ml kg^{-1}) or porcine relaxin (5 or 50 μg kg^{-1}) or salbutamol (100 or 500 μg kg^{-1} ; Glaxo plc, Ware, Herts, UK). Rats were killed by an overdose of anaesthetic at 1, 2, 5, 10, 20, 40 or 60 min after treatment and uteri were rapidly removed.

The second series of experiments assessed the effects of glibenclamide on the response to relaxin. Rats were re-anaesthetized and given an infusion of glibenclamide (20 mg kg^{-1}) or vehicle (0.02 mol sodium hydroxide l^{-1} in 4% glucose, w/v, 5 ml kg^{-1}) 24 h after initial surgery over 5 min. This dose of glibenclamide was sufficient to produce greater than 19-fold antagonism of relaxin as a uterine relaxant (Downing & Hollingsworth, 1991). A bolus injection of relaxin (50 μg kg^{-1}) or saline (1 ml kg^{-1}) was given 15 min later; rats were killed by anaesthetic overdose 2 min later and uteri were rapidly removed.

The third series of experiments was to determine the effect of the development of tolerance to relaxin on relaxin-bolus-induced changes in uterine cAMP concentrations. After initial surgery and recovery for 24 h, conscious rats were infused with relaxin (50 μg $kg^{-1} h^{-1}$) or saline (0.15 ml h^{-1}) for 40 h. Rats were subsequently injected with saline (1 ml kg^{-1}), relaxin (50 μg kg^{-1}) or salbutamol (100 μg kg^{-1}) to achieve the following combinations: (i) saline infusion and saline bolus injection; (ii) saline infusion and relaxin bolus injection; (iii) saline infusion and salbutamol bolus injection; (iv) relaxin infusion and relaxin bolus injection. Rats were killed 2 min after bolus injection and uteri were rapidly removed.

cAMP concentrations

Uteri were immediately frozen in liquid nitrogen and then homogenized in 4 ml 5% (w/v) trichloroacetic acid. The need for rapid dissection did not allow the removal of the endometrium. The homogenate was centrifuged at 1200 g and the supernatant extracted four times with 4 ml diethylether to remove the trichloroacetic acid. Four aliquots of the supernatant, two of 25 μl and two of 50 μl , from each sample of the aqueous extract were assayed for cAMP by radioimmunoassay (Amersham cAMP [3H] assay system). The interassay and intra-assay coefficients of variation were 4.1% ($n = 4$) and 4.5% ($n = 12$), respectively. The minimum sensitivity was 0.25 pmol. After centrifugation the pellet was solubilized in 2 ml 4 mol sodium hydroxide l^{-1} . Four aliquots of the solubilized pellet, two of 1 μl and two of 2 μl , were assayed for protein concentration by the Lowry–Folin method (Lowry *et al.*, 1951). The concentrations of cAMP are given as pmol kg^{-1} protein.

Uterine contractions

Nonpregnant rats were anaesthetized with tribromoethanol (240 mg kg^{-1} , i.p.), bilaterally ovariectomized and equipped with a jugular vein cannula and an intrauterine balloon (Downing *et al.*, 1989). All rats were given morphine sulfate (5 mg kg^{-1}) for post-operative analgesia. Uterine contractions were recorded from 24 h after surgery for 60 min before and for 60 min after bolus i.v. injection of relaxin (5 or 50 μg kg^{-1}) or salbutamol (100 or 500 μg kg^{-1}). Uterine contractions were analysed as integral of area under the pressure curve in 5 min intervals after bolus dose of relaxant and expressed as percentage inhibition compared with the mean integral of the 12 $\times$ 5 min intervals before bolus dose.

Preparation of drugs

Porcine relaxin was isolated and purified from pregnant sow ovaries by the method of Sherwood & O'Byrne (1974). Relaxin was bioassayed *in vitro* by inhibition of electrically stimulated uterus from oestrogen-treated rats (100 µg oestradiol benzoate kg⁻¹ s.c.), using porcine relaxin, kindly donated by O. D. Sherwood (Champaign, Illinois, USA), as standard. Our relaxin preparation was equipotent with highly purified relaxin. Latex for the manufacture of intrauterine balloons was kindly provided by Revertex plc (Harlow, Essex, UK) and glibenclamide by Hoescht Ltd (Hounslow, UK).

Statistical analysis

Data are given as means ± SEM. Statistical analysis of cAMP concentrations was by two-way analysis of variance and the least significant difference test.

Results

Salbutamol bolus dose

Salbutamol (100 and 500 µg kg⁻¹) produced an initial inhibition of uterine contractions of about 80% which was maintained between 50 and 90% with the higher dose, but declined to about 30% inhibition over 60 min with the lower dose (Fig. 1a, b). Salbutamol (100 and 500 µg kg⁻¹) injection resulted in an initial 19-fold increase in uterine cAMP concentrations (Fig. 1c, d). Uterine cAMP concentrations in rats treated with salbutamol (100 and 500 µg kg⁻¹) were significantly greater than those in saline-treated rats for up to 5 and 10 min, respectively, after injection.

Fig. 1. Effect of (a) 100 and (b) 500 µg salbutamol kg⁻¹, i.v. (■) on uterine contractions and, in a separate group of rats, on uterine cAMP concentrations, (c) and (d), respectively. Saline-injected controls for cAMP measurements are shown (□). Ordinates are % reduction in integral of uterine contractions (a, b) and uterine cAMP concentrations (c, d). Values are means ± SEM, *n* = 4–5. **P* < 0.05 versus control for cAMP measurements (two-way ANOVA).

Relaxin bolus dose

Relaxin ($5 \mu\text{g kg}^{-1}$) initially produced about a 70% inhibition of uterine contractions which declined to 30% inhibition by 60 min after injection (Fig. 2a). Relaxin ($50 \mu\text{g kg}^{-1}$) resulted in an immediate 90% inhibition of uterine contractions which was well maintained and had fallen to only about 70% inhibition by 60 min (Fig. 2b). Uterine cAMP concentrations were unchanged by relaxin ($5 \mu\text{g kg}^{-1}$; Fig. 2c). Uterine cAMP concentrations in rats given relaxin ($50 \mu\text{g kg}^{-1}$) were only significantly greater than those in uteri from rats given saline for 1–5 min (1.7- to 3.2-fold) after injection.

Fig. 2. Effect of (a) 5 and (b) $50 \mu\text{g relaxin kg}^{-1}$, i.v. (■) on uterine contractions and, in a separate group of rats, on uterine cAMP concentrations, (c) and (d), respectively. Saline-injected controls for cAMP measurements are shown (□). Ordinates are % reduction in integral of uterine contractions (a, b) and uterine cAMP concentrations (c, d). Values are means $\pm$ SEM, $n = 4-7$. * $P < 0.05$ versus control for cAMP measurements (two-way ANOVA).

Glibenclamide–relaxin interaction

Uterine cAMP concentrations were significantly greater ($P < 0.025$) 2 min after relaxin ($50 \mu\text{g kg}^{-1}$) bolus dose treatment ($13.7 \pm 2.2 \text{ pmol mg}^{-1} \text{ protein}$, $n = 5$) than after saline bolus injection ($6.7 \pm 0.6 \text{ pmol mg}^{-1} \text{ protein}$, $n = 7$) in rats infused with glibenclamide vehicle. Relaxin ($50 \mu\text{g kg}^{-1}$) was still able significantly ($P < 0.05$) to increase cAMP concentrations ($11.0 \pm 1.4 \text{ pmol mg}^{-1} \text{ protein}$, $n = 6$) in animals infused with glibenclamide.

Relaxin-tolerant animals

Twenty-four hours after initial surgery, relaxin bolus ($50 \mu\text{g kg}^{-1}$) increased uterine cAMP concentrations 2 min after injection (Table 1). However, relaxin bolus ($50 \mu\text{g kg}^{-1}$) failed to increase cAMP concentrations after either saline or relaxin infusion for 40 h. Bolus injection of salbutamol ($100 \mu\text{g kg}^{-1}$) could produce a large increase in uterine cAMP concentrations after 40 h of saline infusion (Table 1) of a similar magnitude to that observed in noninfused rats (Fig. 1c).

Table 1. Effect of bolus i.v. injection of relaxin ($50 \mu\text{g kg}^{-1}$), salbutamol ($100 \mu\text{g kg}^{-1}$) or saline (1 ml kg^{-1}) on uterine cAMP concentrations in rats not infused or rats infused with saline (0.15 ml h^{-1}) or relaxin ($50 \mu\text{g kg}^{-1} \text{ h}^{-1}$) for 40 h

Infusate	Bolus treatment	cAMP concentrations ($\text{pmol mg}^{-1} \text{ protein}$)
None	Saline	7.7 ± 0.9 (5) ^a
None	Relaxin	13.4 ± 2.5 (6) ^a
Saline	Saline	8.6 ± 0.9 (5) ^b
Saline	Relaxin	9.2 ± 0.7 (7)
Relaxin	Relaxin	11.4 ± 1.5 (10)
Saline	Salbutamol	89.7 ± 13.7 (6) ^b

Uterine cAMP concentrations were measured 2 min after bolus injection. Groups with the same superscripts were compared and are significantly different (^a $P < 0.05$; ^b $P < 0.005$).

Discussion

Salbutamol (100 and $500 \mu\text{g kg}^{-1}$ i.v.) produced large rises (19-fold) in uterine cAMP concentrations and induced prolonged inhibition of spontaneous uterine contractions. These observations are compatible with the suggestion that these two events are causally related (Johansson & Andersson, 1980; Hsu *et al.*, 1985; Sanborn & Anwer, 1990). We have shown that the two events are well correlated. However, cAMP concentrations had declined to basal values while there was still significant mechanical inhibition. It is possible that after initial uterine inhibition in response to cAMP-mediated events, signal transduction mechanisms other than those involving cAMP are important for maintained uterine relaxation to salbutamol. It should be noted, however, that similar declines in cAMP concentrations to basal values before termination of functional effects are commonly seen in other tissues (Adelstein, 1980). Such dissociations have been explained by persistent activation of enzymes subsequent to adenyl cyclase in the biochemical cascade leading to the final functional events.

Relaxin ($5 \mu\text{g kg}^{-1}$) had no effect on cAMP concentrations and relaxin ($50 \mu\text{g kg}^{-1}$) produced only a small (2–3-fold) increase in cAMP concentrations of short duration after i.v. administration. This latter change was much less than that observed with salbutamol at doses that produced equivalent and marked inhibition of uterine contractions. It is reasonable to conclude that a rise in cAMP concentrations is not the essential mediator of relaxin-induced inhibition of uterine contractions in rats *in vivo*. The observation that relaxin could still increase cAMP concentrations to a similar extent after a dose of glibenclamide that reduced the uterine relaxant action of relaxin (Downing & Hollingsworth, 1991) provides further support for the dissociation of the two events.

We intended to observe whether this small rise in uterine cAMP concentrations to relaxin ($50 \mu\text{g kg}^{-1}$) was still observed in rats that had been made tolerant to relaxin by its infusion ($50 \mu\text{g kg}^{-1} \text{ h}^{-1}$ for 40 h; Downing & Hollingsworth, 1989). However, relaxin bolus no longer produced any change in uterine cAMP concentrations after 40 h of saline infusion. It is possible that uterine responses to relaxin were diminished 40 h after ovariectomy in saline-infused rats owing to a decline in numbers of relaxin receptor as a consequence of lack of oestrogen (Mercado-Simmen *et al.*, 1982). Relaxin still produced a uterine relaxant effect after saline infusion (Downing & Hollingsworth, 1989). Salbutamol could still elicit a large rise in uterine cAMP concentrations after saline infusion for 40 h. These data provides additional support for the hypothesis that a rise in cAMP concentrations and relaxation to relaxin are not fundamentally linked.

It is possible that uterine phosphodiesterases degraded significant amounts of cAMP during the dissection stage. However, this explanation is unlikely as large rises in cAMP concentrations were observed after salbutamol administration. We had deliberately not treated the rats or the tissues with phosphodiesterase inhibitors as we wished to observe effects in their absence. There are two possible explanations for the small rise in cAMP concentrations detected after relaxin ($50 \mu\text{g kg}^{-1}$). cAMP might play a minor, transitory role in relaxin-induced mechanical inhibition that is maintained by other mechanisms. Alternatively, the rises in cAMP concentrations may have occurred in the endometrium, as it has been shown *in vitro* that human relaxin can augment cAMP concentrations in human endometrium in culture (Chen *et al.*, 1988; Fei *et al.*, 1990).

If cAMP does not play a significant role in signal transduction in the myometrium with relaxin *in vivo*, it is necessary to consider other mechanisms. Downing & Hollingsworth (1991) have shown that relaxin, as a uterine relaxant, was selectively antagonized by the sulphonylurea glibenclamide. It is possible that this antagonism results from the ATP-sensitive potassium channel blocking properties of glibenclamide (Sturgess *et al.*, 1988). If so, relaxin may act via opening of potassium channels in the plasmalemma. However, glibenclamide has several metabolic effects on non-pancreatic tissues (Smith, 1990; Farese *et al.*, 1991) that could explain this antagonism. Recent work has shown that relaxin, as a relaxant in the isolated uterus of the rat, is not antagonized by glibenclamide (Hughes *et al.*, 1992). The conclusion from this latter study is that either relaxin acts by different mechanisms *in vitro* and *in vivo* or the antagonism of relaxin by glibenclamide *in vivo* was not a consequence of the ability of the latter compound to block ATP-sensitive potassium channels.

In summary, relaxin produces only a small rise in cAMP concentrations in rat uteri *in vivo*. The rise in cAMP concentrations may not be the mechanism by which uterine relaxation is achieved.

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