Effects of porcine relaxin on contraction, membrane response and cyclic AMP content in rat myometrium in comparison with the effects of isoprenaline and forskolin

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1 The longitudinal muscle from the uterus of oestrogen-treated rats was quiescent in Mg-free Krebs solution. Electrical stimulation generated phasic contraction, which was depressed to 35% and 18% by 50 mu and 150 mu porcine relaxin, respectively.

2 The phasic contractions were more strongly depressed to 26% by 50 mu relaxin in solution containing 0.6 mM Mg, and the depression lasted for more than 4 h after the removal of relaxin. During the persisting depression, raising the external Ca to 7.5 mM did not restore the contraction, but the contraction was restored by removal of Mg.

3 The depression of the phasic contraction by relaxin, examined in Mg-free solution, was enhanced and reduced by pretreatment of the tissue with 0.6 mM Mg and 0.6 mM Mn, respectively, for about 15 min. In contrast, the depression of contraction by isoprenaline or forskolin was enhanced by pretreatment with either Mg or Mn.

4 The cellular content of cyclic AMP was measured in Krebs solution containing 0.6 mM Mg. The values were 1.24 (pmol mg⁻¹ protein) in control solution, and 2.31 and 1.56 when the tissues were treated with 150 mu relaxin and 10⁻⁹ M isoprenaline, respectively.

5 The cyclic AMP production in response to 10⁻⁷ M forskolin measured in Mg-free solution was enhanced when the tissue was pretreated with either 0.6 mM Mg or Mn for 15 min. The cyclic AMP production in response to 100 mu relaxin was increased when the tissue was pretreated with 0.6 mM Mg, and was unchanged by pretreatment with Mn. The cyclic AMP production in response to 10⁻⁹ M isoprenaline was unchanged by pretreatment with the divalent cations.

6 The membrane potential of the muscle was -60.8 mV in Krebs solution containing 0.3 mM Mg, and electrical stimulation induced an action potential which consisted of spike and plateau components. Application of 150 mu relaxin reduced the duration of the plateau; the contractions were progressively depressed. The resting membrane potential and membrane resistance were unchanged by application of 150 mu relaxin. The membrane was hyperpolarized by 2.8 mV, accompanied by a decrease in membrane resistance, when 10⁻⁹ M isoprenaline was applied.

7 Although there were several differences between the effects of relaxin and isoprenaline, it is probable that some process, which is cyclic AMP-dependent, accelerated by Mg and depressed by Mn, is involved in the depressant action of relaxin on contraction.

Keywords: Relaxin; isoprenaline; forskolin; Mg; Mn; cyclic AMP; myometrium

Introduction

Relaxin, a peptide hormone produced mainly in the ovary, has several target tissues, including the myometrium on which it exerts a relaxant effect (rat: Sawyer *et al.*, 1953; Bradshaw *et al.*, 1981; mouse: Wqvist, 1959; man: Szlachter *et al.*, 1980; guinea-pig: Porter, 1972; mini-pig: Porter & Watts, 1986). It was recently reported that porcine relaxin specifically depressed the plateau component of the action potential in the myometrium of the pregnant and oestrogen-treated rat, thereby inhibiting the contraction (Chamley & Parkington, 1984). It was further shown that the cellular adenosine 3',5'-cyclic monophosphate (cyclic AMP) was increased by relaxin in dispersed myometrial cells in culture, as with isoprenaline (Hsu *et al.*, 1985).

The relaxant effect presumably depends on the population of relaxin receptors in the surface membrane. Relaxin receptor population in rat myometrium was shown to change during pregnancy, its peak being attained on day 17 of pregnancy (Mercardo-Simmen *et al.*, 1980; 1982). In addition, the binding of relaxin to the membrane is accelerated when Mg, Ca or Mn is present in the incubation media (Mercardo-Simmen *et al.*, 1980).

The aim of the present experiments was to obtain further insight into relaxin action in comparison with β -adrenoceptor

effects. For this purpose, the effects of porcine relaxin were examined mainly on isolated longitudinal muscle strips in oestrogen-primed rat uterus. The influence of Mg and Mn on the relaxant effects of relaxin was investigated, since these ions are known to modify the adenylate cyclase activity in cell-free myometrium (Krall *et al.*, 1988; Arkinstall & Jones, 1990). Secondly, the effects of relaxin, isoprenaline and forskolin on cellular cyclic AMP production were compared. The results indicated several differences between the effects of relaxin and β -adrenoceptor agonists, leading to the conclusion that some cyclic AMP-dependent process, which is accelerated by Mg and decelerated by Mn, is involved in the depressive action of relaxin on rat myometrial contraction.

Methods

Preparations

Wistar-strain female rats (200–250 g) were ovariectomized under pentobarbitone anaesthesia and about 2 weeks later were given a single subcutaneous injection of 50 μ g oestradiol-17 β -benzoate dissolved in sesame oil. Ovariectomized rats are called 0E, and those injected with oestradiol and left for 24, 48 and 72 h are called 1E, 2E and 3E, respectively. Animals were mainly used 72 h after oestrogen treatment (3E). In other

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experiments, in which 17 day-pregnant rats were used, pro-oestrus rats were housed with males overnight and day 1 of pregnancy was taken as the day on which copulatory plugs were found in the vagina.

Animals were stunned and bled, and uterine horns removed. The uterus was cut open along the mesometrium, and the endometrium was removed carefully with jeweler's forceps. Longitudinal and circular muscle strips (0.5 mm wide, 5 mm long) were dissected from the ovarian portion of the uterus. Muscle strips were mounted vertically in an organ bath. One end was fixed and the other end was connected to a force transducer. The bathing solution ($36.0 \pm 0.5^\circ\text{C}$) flowed continuously at a rate of 8 ml min^{-1} , and the capacity of the organ bath was 0.2 ml. Platinum electrodes were placed near the ends of the muscle strip, and electric pulses, 1 s duration and supramaximal intensity, were applied. The preparation was equilibrated with warm control solution for 1–2 h before the experiment began.

For measurement of electrical activity, the muscle strip was mounted in a partition chamber (1 ml in volume) as described by Abe & Tomita (1968), and superfused at a rate of 5 ml min^{-1} . Electrical activity of the muscle cell was recorded intracellularly with glass microelectrodes filled with 3M KCl (30–50 M Ω in resistance). For the recording of electrotonic potentials, the microelectrode was placed 0.2 mm from the stimulating electrode. For simultaneous records of electrical and mechanical activity, one end of the muscle strip was tied with a thread and connected to a force transducer. Signals were amplified and recorded by an ink-writing pen recorder. Membrane responses were in some cases stored on a data recorder and reproduced at appropriate sweep speed and magnitude.

Measurements of contraction and membrane response are expressed as mean $\pm$ s.d.

Solutions and drugs

The control solution (Mg-free Krebs solution) had the following composition (mm): NaCl 123.1, NaHCO₃ 15.5, KCl 4.7, KH₂PO₄ 1.2, CaCl₂ 2.5, and glucose 11.5. It was equilibrated with a gas mixture of 95% O₂ + 5% CO₂ (pH of 7.4). A solution of MgCl₂ or MnCl₂ (both 1 M) was added to give the desired concentration. To prepare high Ca solution, KH₂PO₄ was omitted to prevent the precipitation of Ca, and NaHCO₃ was decreased to 12 mm, where 4.7 mm NaCl was added to balance the osmolality.

Porcine relaxin (NIH-RXN-P1,3000 guinea-pig units 1 mg^{-1}) was a gift from National Hormone and Pituitary Program, U.S.A. It was dissolved in distilled water and stored at -40°C . Acryl and silicone wares were used at all times when relaxin was used. The concentration of relaxin is expressed in guinea-pig units. Isoprenaline-HCl and forskolin were from Nikken and Sigma, respectively. Stock solution of forskolin was prepared by dissolving it in dimethylsulphoxide (DMSO), so that the final concentration of DMSO in the Krebs solution was at most 0.1%. Molar concentrations of these agents are given. Drugs were added to the perfusion solution and took 30 s and 3 min to reach the tissue in the contraction and microelectrode organ baths, respectively.

Cyclic AMP measurement

The blood attached to the surface of the uterus was removed with a cotton bud prior to preparing muscle strips. In one series of experiments (Table 1), longitudinal muscle strips (1–3 mg wet weight) were incubated in 0.6 mM Mg containing solution for 3 h and were treated with 10^{-9} M isoprenaline or 150 mu relaxin.

In the divalent cation pretreatment experiments (Figure 11), longitudinal muscle strips were incubated in Mg-free Krebs solution for 2 h, and were exposed to 0.6 mM Mg or 0.6 mM Mn for 15 min. Then muscle strips were washed with Mg-free

Krebs solution for 45 min, and muscles were exposed to 10^{-9} M isoprenaline, 10^{-7} M forskolin and 100 mu relaxin for 5, 10 and 10 min, respectively, when cyclic AMP in the cells attained maximal value. At appropriate points in the above sequential procedures, muscle strips were harvested, and were immediately frozen with liquid nitrogen, then lyophilized for 2–3 h. The lyophilized muscles were homogenized with an agate mortar and pestle, after an aqueous solution containing 6% trichloroacetic acid (1.5 ml, 3°C) had been added to terminate the reactions in muscle cells. The homogenate was centrifuged at 2500 g for 15 min at 4°C , and the precipitate was used to determine protein content by the method of Lowry *et al.* (1951). The supernatant was washed 3 times with 5 volumes of water-saturated diethyl ether. The upper ether layer was discarded at each wash. Cyclic AMP content retained in the water layer was measured by a radioimmunoassay system (Amersham, UK), which allows high sensitivity without interference from ATP (Volker *et al.*, 1985).

Measurements are expressed as mean $\pm$ s.e.mean in Figure 11 and Table 1, and statistical significance of differences between means was tested by Student's *t* test.

Results

Hormonal influence on relaxin action

Effects of 150 mu relaxin on the contractile activity of rat myometria were first examined in Mg-free solution (Figure 1). Longitudinal muscle strips excised from ovariectomized rat (OE) exhibited frequent spontaneous contractions. On application of relaxin the baseline tension declined slightly, and the frequency and amplitude of spontaneous contraction decreased slightly. A similar observation was made on the 1E preparations. Spontaneous contraction was absent in the 3E preparations, but phasic contractions could be evoked by electrical stimuli. Relaxin strongly depressed the contraction and recovery from the depression was very slow.

Circular muscle strips of the OE rat were spontaneously active, and relaxin caused slight depression of the phasic contraction. The frequency of spontaneous contractions was less in 1E preparations, and both the frequency and amplitude were slightly diminished when relaxin was applied. Spontaneous activity was absent in 3E preparations, and phasic contraction evoked by electrical stimulation was strongly depressed by relaxin. As with the longitudinal muscle, the recovery from the depressant effect was slow.

Results are summarized in Figure 1b, where the effects of relaxin are indicated by % response of phasic contraction during 8–10 min application of relaxin relative to the mean control amplitude of 5 successive phasic contractions which occurred prior to the application of relaxin. These results indicate that the inhibitory effect of relaxin increased as exposure time to oestrogen increased in both the longitudinal and circular muscle strips.

The effects of 150 mu relaxin on the longitudinal and circular muscle strips excised on day 17 of pregnancy were also examined (Figure 1a, bottom). The longitudinal muscle strips on day 17 of pregnancy were spontaneously active only for about 30 min after being placed in the organ bath, but phasic contractions could be elicited by electrical stimuli. Relaxin (150 mu) did not cause a significant effect on the amplitude of evoked contractions, though the contraction was partly depressed temporarily after relaxin was removed. Relaxin (150 mu) depressed the contraction to $97 \pm 4\%$ ($n = 10$) of the control value in Mg-free solution, and to $91 \pm 13\%$ ($n = 10$) in the solution containing 0.6 mM Mg. Phasic contractions were spontaneously generated in the circular muscle strips excised on day 17 of pregnancy, and relaxin caused slight depression. In all cases, the spontaneous contractions were strongly depressed for about 3 min after relaxin was removed. Relaxin (150 mu) depressed the contraction to $89 \pm 9\%$

Figure 1 (a) Effects of porcine relaxin on the phasic contractions of uterine longitudinal (LM) and circular (CM) muscle strips, examined in Mg-free Krebs solution. Muscle strips were taken from ovariectomized rats at 0 (OE), 1 (1E) and 3 (3E) days after $50 \mu\text{g}$ oestradiol- 17β injection, and on day 17 of pregnancy. Application of relaxin (150 mu) is indicated by bars. Dots indicate electrical stimulation of the muscle. (b) Shows the average inhibition of phasic contractions by relaxin (ordinate; mean $\pm$ s.d.). Figures in parentheses are the number of muscle strips used for measurements; (■—■) longitudinal muscle; (●---●) circular muscle.

($n = 18$) of the control value in Mg-free solution, and to $49 \pm 24\%$ ($n = 9$) in the solution containing 0.6 mM Mg, respectively.

For the sake of simplicity in analyzing the relaxin action, longitudinal muscle strips from rats treated with oestrogen for 3 days (3E) were used in the following experiments.

Effects of Mg and Ca on relaxin action

It has been reported that depressive effects of relaxin on myometrial contraction *in vitro* varied depending on the incubation period with artificial saline media (Wiqvist & Paul, 1955). The influence of incubation and Mg ion contained in the bathing solution were therefore examined. The muscle strips were freshly dissected, and 50 mu relaxin was applied intermittently during 10 h incubation. In the tissue shown in Figure 2, relaxin initially depressed the contractions in Mg-free solution, and the degree of contractile depression became weaker when relaxin was applied about every 2 h. Exchange of the bulk solution in the organ bath took 30 s. However, it took about 6 min to reach the maximum depression. Contractions recovered completely in 10 min on washing with normal solution. In the presence of 0.6 mM Mg (b), the amplitude of the phasic contractions was also depressed by

the first application of 50 mu relaxin, and the recovery in the control solution was incomplete even after by 4 h washing. During the recovery, phasic contractions were again depressed by relaxin, and the minimum amplitude was nearly the same with repeated application of relaxin. The amplitude of phasic contractions was increased when the external Mg was removed, and again depressed when Mg was readmitted (bottom trace). Such a 'persistent' depression of contraction in the presence of Mg was observed in 10 other preparations. In the absence of relaxin, the phasic contraction remained nearly identical in size for 10 h in the solution containing 0.6 mM Mg.

Experiments similar to those shown in Figure 2 were carried out on different muscle strips taken from different animals, and representative results are illustrated graphically in Figure 3. From results from 12 preparations studied in Mg-free solution, responses can be classified into 3 types. In 6 preparations out of 12, designated type i, the depressive effect of relaxin was progressively reduced; in type ii, the degree of depression by relaxin was nearly identical throughout 9 h incubation; in type iii which was observed only once, the depression was initially weak, and became stronger as the incubation time increased. In solution containing 0.6 mM Mg (Figure 3b), the mean of 5 successive phase contractions before the first application of relaxin was taken as 100%. The 3 different patterns of response in terms of incubation time were also observed. Only in one preparation out of 11 muscle strips examined, was the depressive effect reduced after 9 h incubation (b(i)). In 10 preparations, the degree of contractile depression by relaxin was nearly steady after 3 h incubation (b(ii); b(iii)). The % contraction estimated at the second application of relaxin (3 h incubation) was $35 \pm 16\%$ ($n = 12$) in Mg-free solution, and $26 \pm 18\%$ ($n = 11$) in the solution containing 0.6 mM Mg. Comparison of Figures 3a and b suggests that external Mg is needed to keep the relaxation action stable *in vitro* for several hours.

An explanation of the persistent effect of relaxin is that a stabilizing action of Mg was enhanced by relaxin. The effects of raising the concentration of Ca on relaxin depression were examined in the absence and presence of Mg (Figure 4). The amplitude of evoked phasic contractions was enhanced when the external Ca concentration was raised to 7.5 mM (Figure 4a). About 7 min was needed to reach the maximal amplitude. The effect was the same when Ca was applied 30 min after application of 50 mu relaxin in the Mg-free solution. Mg, 0.6 mM , depressed the phasic contractions, but their amplitudes were restored by application of Ca, 7.5 mM (b). The contractions were strongly depressed in the presence of 0.6 mM Mg by applying 50 mu relaxin, and the persisting depression lasted for about 5 h. The amplitude of the depressed contractions was increased only slightly by 7.5 mM Ca during the initial 2 h after removal of relaxin. Thereafter contractions of large amplitude occurred on exposure to 7.5 mM Ca. It took about 10 min after each admission of 7.5 mM Ca to produce full-size phasic contractions. Removal of external Mg also restored the phasic contraction (bottom trace).

A question arising from the foregoing results is why the recovery was incomplete after the removal of relaxin when Mg was in the perfusing solution. Furthermore, is the responsiveness of muscles to relaxin changed during preparation (30 min) in Mg-free solution at room temperature ($20\text{--}27^\circ\text{C}$)? Figure 5 demonstrates the effect of brief treatment with Mg and of cooling on the depression produced by 50 mu relaxin. The amplitude of contraction was depressed by the first application of relaxin (a). The muscle was treated with 0.6 mM Mg for 15 min, then the inhibition by relaxin was stronger. When the muscle was cooled down to 24°C for 40 min, the baseline tension rose and the phasic contraction became smaller (b). When the muscle was rewarmed to 36°C , the baseline tension declined, phasic contractions were restored, and the depressive effect by relaxin became very small. The muscle was again treated with Mg, the depressive effect of relaxin returned (c). From these results, it is suggested that pretreatment with Mg produced a residual effect on the muscle to cause stronger

Figure 2 Effects of relaxin on the phasic contractions of uterine longitudinal muscle of oestrogen-treated rats (3E). The 2 muscle strips were excised from different animals; (a) responses to application of 50 μ u relaxin (bars) examined in Mg-free solution. (b) Sequential responses examined in solution containing 0.6 mM Mg. Phasic contractions were evoked by supramaximal electrical stimulation at 90 s intervals. Figures in parentheses show the time in min after mounting the muscle strip in the warm organ bath.

contractile depression by relaxin, and the effect of Mg could be removed by low temperature.

Relaxin action on membrane responses

In order to correlate electrical and mechanical events, the membrane activity and phasic contraction were recorded

simultaneously, and effects of relaxin were examined in Krebs solution containing 0.3 mM Mg. The preparations were quiescent, and the resting membrane potential was -60.8 ± 4.1 mV (mean $\pm$ s.d., $n = 41$). Activity was generated by electrical pulses which produced a large action potential with a plateau upon which spikes were superimposed (Figure 6). The duration of the action potential decreased gradually when 50 μ u

Figure 3 Changes with time of the inhibitory effects of relaxin on the phasic contractions in the absence (a) and presence (b) of 0.6 mM Mg. Longitudinal muscle strips freshly excised were incubated in warm solution for 1 h, and 50 μ u relaxin was applied about every 2 h thereafter (abscissae). In (a) the minimum amplitude during relaxin application is expressed as a % of the phasic contraction immediately prior to the application; whereas in (b) all inhibitions are expressed relative to the control response after the initial 1 h incubation. In both (a) and (b), the responses are categorized into 3 types (i, ii, iii), and the occurrence in the total preparations examined are indicated in parentheses.

Figure 4 Effects of high Ca (7.5 mM) on the depression of contractions by relaxin, examined in the absence (a) and presence (b) of 0.6 mM Mg. Application of 7.5 mM Ca, 50 μ M relaxin and 0.6 mM Mg are indicated by broken lines above, bars below and arrows, respectively. Successive records are shown in (b). Figures in parentheses indicate the time in min after removal of relaxin.

relaxin was applied, and accordingly the duration of the phasic contraction became shorter, then the amplitude became reduced. The depression of both membrane and contractile activities was still apparent 20 min after the removal of relaxin (Figure 6b). When Mg was omitted from the bathing solution, the action potential became prolonged, the contraction became larger, and spontaneous activity occurred. On re-exposure of the muscle to 0.3 mM Mg and 150 μ M relaxin the action potentials were not much depressed, whereas the contractions gradually decreased. When relaxin was removed, the amplitude of contraction gradually increased, while the action potentials were not much altered (Figure 6d). The resting membrane potential remained unchanged throughout.

The effects of relaxin on membrane response were compared in the presence of 0.6 mM Mg with those of isoprenaline which also caused depression. The concentrations of the agents were chosen so that they depressed the phasic contraction to a similar degree (Table 1). In the cell illustrated in Figure 7, the membrane potential was -59 mV, and application of 10^{-9} M isoprenaline hyperpolarised the membrane by 3.5 mV (a), and reduced the amplitude of the electrotonic potential by 5% (ai, aii). From different muscle strips, the mean membrane hyperpolarization and the reduction of electrotonic potential by 10^{-9} M isoprenaline were 2.8 ± 0.3 mV ($n = 9$) and $4.2 \pm 1.2\%$, respectively. The membrane potential and the electrotonic potential were not changed by 150 μ M

Figure 5 Effects of low temperature on the interaction between Mg and relaxin actions. The muscle was perfused with Mg-free Krebs solution (36°C), and 50 μ M relaxin and 0.6 mM Mg were applied during the periods indicated by bars and arrows, respectively. The muscle was cooled to 24°C during the period indicated by the broken line. Successive records are shown.

Figure 6 Effects of relaxin on the intracellularly recorded membrane activity (upper traces) and simultaneously recorded contraction (lower traces), examined in the presence of 0.3 mM Mg. The muscle was stimulated by electrical pulses at intervals of 1 min. All electrical records were obtained from the same cell. Record (b) shows the activities 20 min after relaxin was removed, and Mg was omitted during the period indicated by the broken line. Relaxin and Mg were applied or removed 3 min before the time indicated by signals, because of slower exchange of the solutions in the organ bath.

relaxin ($n = 7$), indicating that isoprenaline but not relaxin alters passive resting membrane properties.

Effects of Mn on relaxin action in comparison with β -adrenoceptor stimulation

It has been reported that in cell-free systems the increase in adenylate cyclase activity linked to β -adrenoceptor activation is facilitated by adding Mn (myometrium, Krall *et al.*, 1988; ocular ciliary process membrane, Mittag *et al.*, 1988). It has also been shown that relaxin increased the cellular content of cyclic AMP in myometrial cells in culture (Hsu *et al.*, 1985). The question arises as to whether or not the treatment of intact muscle with Mn affects the cyclic AMP-mediated response. In the experiment shown in Figure 8, the influence of Mn on the effects of isoprenaline and relaxin was studied. Application of 3×10^{-10} M isoprenaline and 50 mu relaxin in Mg-free solution depressed the phasic contractions to 39% and 47% of the control value, respectively. Contractions were

strongly depressed by 0.6 mM Mn and recovered on removal of Mn. After this procedure, contractions were depressed by 3×10^{-10} M isoprenaline more strongly than before, whereas the depression by 50 mu relaxin became markedly reduced. The muscle was then exposed to 2 mM Mg for 1 h, and the evoked contractions were strongly depressed. Phasic contractions gradually developed when Mg was removed until the amplitude recovered almost to the previous control level. Contractions were then strongly depressed by 50 mu relaxin. Thus, as with Mg, Mn pretreatment caused a residual influence in the muscle so that the inhibitory effects of isoprenaline and relaxin were affected in opposite directions.

Forskolin is an agent which activates the catalytic unit of adenylate cyclase (Seamon *et al.*, 1981), and increases intracellular cyclic AMP concentration in uterine smooth muscle (Marshall & Fain, 1984). The inhibitory effect of forskolin on myometrium was shown to be enhanced when the muscle was pretreated by Mg (Maruta & Osa, 1989). The effects of Mn on the action of 10^{-7} M forskolin were therefore examined in

Table 1 Effects of relaxin (150 mu) and isoprenaline (10^{-9} M) on phasic contractions and tissue cyclic AMP content in the longitudinal muscle from oestrogen-primed rat uterus, examined in Krebs solution containing 0.6 mM Mg

	Control	Relaxin	Isoprenaline
Time s to reach the minimum contraction		$368 \pm 55^*$ ($n = 8$)	172 ± 45 ($n = 8$)
Amplitude of contraction (% of control)		16 ± 8.3 ($n = 8$)	21 ± 8.7 ($n = 8$)
Cyclic AMP (pmol mg $^{-1}$ protein)	1.24 ± 0.24 ($n = 9$)	$2.31 \pm 0.25^*$ ($n = 9$)	$1.56 \pm 0.28^*$ ($n = 8$)

The phasic contractions were generated every 60 s by electric pulses, and the time in s to reach the minimum value of phasic contraction was determined by the time after the exchange of bath solution. They were significantly different from each other. The minimum amplitude of contraction during exposure is expressed as a percentage of the mean of amplitude of 5 successive contractions immediately before the drug application. Cyclic AMP content was determined after 3 min and 6 min exposure to isoprenaline and relaxin, respectively and were significantly different from the control. Values are means $\pm$ s.d. n = number of preparations. Statistical analysis for the significance of differences between means was by Student's *t* test: * $P < 0.02$.

Figure 7 Effects of isoprenaline (a) and relaxin (b) on the membrane potential and membrane resistance examined in the presence of 0.6 mM Mg. Upper and lower traces show current monitor and membrane potential, respectively. Inward current pulses (500 ms) at constant intensity were applied at intervals of 20 s. Magnified electrotonic potentials with a faster sweep are reproduced in a(i) and a(ii) (10 s before and 35 s after the application of isoprenaline), and b(i) and b(ii) (15 s before and 120 s after the application of relaxin). The muscle was washed with Krebs solution for 20 min, after application of isoprenaline. All records were obtained from the same cell.

Mg-free Krebs solution. The phasic contractions were depressed only slightly by 0.1% DMSO, the concentration present in a 10^{-7} M solution of forskolin. Forskolin gradually depressed the phasic contractions, up to 8 min, when the maximal inhibition was reached (Figure 9). Following application of Mn (0.4 mM) for 15 min, and 40 min wash in control solution, forskolin caused a stronger depression of evoked contraction. The enhanced depression lasted more than 3 h after the removal of Mn, though the degree was slightly reduced.

The fact that the inhibitory action of isoprenaline and forskolin was enhanced, whereas that of relaxin was reduced by pretreatment of the muscle strip with Mn, was further investi-

gated. Pairs of muscle strips freshly excised were incubated in the same organ bath with Mg-free Krebs solution, and the effects of agents on the phasic contractions were examined 2 h later. The contractions of agents were adjusted so that the contractile inhibition was not too strong for isoprenaline (3×10^{-10} M) and forskolin (10^{-7} M), and moderate inhibition (100 mu) was caused by relaxin. After 45 min in Mg-free Krebs solution, 0.6 mM was applied for 15 min, and the depressive effect of Mn was examined. The effects of agents were again examined 40 min after the removal of Mn. Figure 10a shows the depression of phasic contractions by isoprenaline examined in 7 muscle strips. The amplitude of the evoked contraction was measured 5 min after the administration of

Figure 8 Effects of Mn and Mg on depression of contraction produced by isoprenaline and relaxin. The muscle strip was perfused with Mg-free Krebs solution, and 3×10^{-10} M isoprenaline and 50 mu relaxin were applied during the periods indicated by broken lines and bars, respectively. Application and removal of 0.6 mM Mn and 2 mM Mg are indicated by arrow heads and arrows, respectively. Sequential records from top to bottom.

Figure 9 Effects of Mn on the inhibitory action of forskolin. The muscle strip was perfused with Mg-free Krebs solution, and 10^{-7} M forskolin was applied during the periods indicated by bars below. Dimethylsulphoxide (DMSO, 0.1%), the vehicle, was applied during the period indicated by broken lines above. Times in min after removal of Mn are indicated in parentheses.

isoprenaline, and the contractile inhibition was expressed as a % of the control and ranged between 100% and 17%. The depression by isoprenaline was enhanced by Mn in all muscle strips examined. In the case of forskolin, maximum inhibition was measured 10 min after addition of the agent. The degree of the contractile depression caused by forskolin was less variable for 7 muscle strips (Figure 10b). Again, the depression by forskolin became enhanced after pretreatment with Mn. In the case of 100 μ relaxin, the degree of contractile depression was measured for the evoked contraction generated 10 min after the administration of the agent (Figure 10c). The degree of the contractile inhibition was always smaller after pretreatment with Mn. The averages of inhibition expressed as a %

response of the amplitude of control were $58 \pm 38\%$ ($n = 7$) and $8 \pm 6\%$ for isoprenaline, $88 \pm 8\%$ ($n = 7$) and $19 \pm 6\%$ for forskolin, $43 \pm 22\%$ ($n = 8$) and $72 \pm 32\%$ for relaxin, before and after the Mn pretreatment, respectively. These results therefore suggest that the intracellular signal transduction system could be different in the case of relaxin, although intracellular cyclic AMP is increased by this agent (Hsu *et al.*, 1985).

Influence of Mg and Mn on cyclic AMP content

Since isoprenaline and relaxin both depress the contractions, their effects on the cellular content of cyclic AMP were com-

Figure 10 Effects of 3×10^{-10} M isoprenaline (a, Iso), 10^{-7} M forskolin (b, For) and 100 μ relaxin (c, Rel) on the phasic contractions, examined before and after the exposure to 0.6 mM Mn. Experiments were carried out in Mg-free Krebs solution. The phasic contractions were generated by electric pulses, and the minimum amplitude of phasic contraction during exposure to agents is expressed as a percentage of the mean of 5 successive contractions immediately before the drug application. The application of agents and Mn is indicated by broken lines and bars. Abscissae indicate the time (in min) after freshly excised muscle strips were exposed to warm Krebs solution (Mg-free). Responses in the same muscle strip are connected by lines.

pared in the presence of 0.6 mM Mg. A higher concentration of isoprenaline (10^{-9} M) was used in the present case, because the change in cyclic AMP content due to 3×10^{-10} M isoprenaline application was hard to detect. It was found that the time for relaxin and isoprenaline to produce maximum depression after the agents were perfused in the bulk solution was different (Table 1). In order to minimize the time resolution, the phasic contractions were generated every 60 s. The mean times in seconds to reach the maximum depression were 368 s ($n = 8$) and 172 s ($n = 8$) for relaxin (150 mu) and isoprenaline (10^{-9} M), respectively. Contractions were depressed to almost the same extent by these doses of the agents. The slower time course of relaxation by relaxin does not appear to be due to diffusion in the extracellular space, because a similar relaxation was reported for a single rat myometrial cell in culture (Hsu & Sanborn, 1986).

Because of the different times to reach the maximum depression, the muscle strips (about 1–3 mg) were treated with relaxin for 10 min and with isoprenaline for 5 min, and the cellular content of cyclic AMP was then measured. Values were 2.31 ± 0.25 pmol mg $^{-1}$ protein ($n = 9$) for relaxin and 1.56 ± 0.28 pmol mg $^{-1}$ protein ($n = 8$) for isoprenaline; cyclic AMP content was 1.24 ± 0.24 pmol mg $^{-1}$ protein ($n = 9$) in non-treated muscle, and the difference from the control value was statistically significant for relaxin ($P < 0.02$).

In relation to the contractile investigation shown in Figure 10, effects of the pretreatment with 0.6 mM Mg or 0.6 mM Mn on cellular cyclic AMP content were examined (Figure 11). Several muscle strips, each weighing about 1–3 mg wet weight, were taken from a uterine horn, and subjected to sequential

procedures. In order to minimize possible differences due to individual animals (cf. Figure 10), the value in a muscle strip exposed to the control solution was taken as 100% for all other muscle strips taken from the same animal. The average control value of cyclic AMP measured in Mg-free solution for 52 muscle strips excised from 37 rats was 0.98 ± 0.02 pmol mg $^{-1}$ protein (mean $\pm$ s.e.mean). The cyclic AMP content was increased (1.17 ± 0.06 pmol mg $^{-1}$ protein, $n = 18$) by exposure of the tissues to 0.6 mM Mg, and the level remained elevated during a 45 min rinse with the control solution. The muscle strip was rapidly frozen by liquid nitrogen 5 min after exposure to isoprenaline, and 10 min after exposure to forskolin or relaxin. The cyclic AMP content in response to either forskolin or relaxin was increased more following pretreatment with 0.6 mM Mg than in tissues kept in Mg-free solution throughout (Figure 11a). In the case of Mn, the cyclic AMP content was significantly increased (1.11 ± 0.06 pmol mg $^{-1}$ protein, $n = 16$) by exposing the tissue to 0.6 mM Mn, but had returned to the control level by 45 min rinse with control solution. The increase in cyclic AMP content in response to forskolin was greater following pretreatment with 0.6 mM Mn, whereas the increase to relaxin was unchanged by pretreatment with Mn (Figure 11b).

The cyclic AMP contents in response to 10^{-8} M isoprenaline, by which evoked contractions were almost abolished, were 1.44 ± 0.07 (pmol mg $^{-1}$ protein, $n = 4$) and 1.66 ± 0.02 ($n = 3$) before and after the treatment with 0.6 mM Mg and 1.42 ± 0.04 ($n = 3$) and 1.54 ± 0.06 ($n = 3$) before and after the treatment with 0.6 mM Mn, respectively.

Discussion

It has been shown that oestrogen treatment is needed to reveal the effect of relaxin by bioassay for either the lysis of the pubic symphysis or the relaxation of the myometrium (for review, see Schwabe *et al.*, 1978; Weiss, 1984). The present results are in agreement with the above finding on the myometrium, and further show that extracellular Mg was not necessary for relaxin to produce strong inhibition in both longitudinal and circular muscles in oestrogen-treated rats. In contrast, in spite of the abundance of relaxin receptors in rat myometrium on day 17 of pregnancy (Mercado-Simmon *et al.*, 1980; 1982), 150 mu porcine relaxin was almost without effect when Mg was absent in the bathing solution. Thus, a different requirement of external Mg for the relaxin-receptor binding is suggested between pregnant and oestrogen-primed rat myometrium. The following discussion will be confined to the action of relaxin on the longitudinal myometrium of the oestrogen-treated rat.

It was demonstrated by Marshall & Kroeger (1973) that the cellular content of cyclic AMP was increased when isoprenaline was administered to rat myometrium. Since then, it has been further proposed that cyclic AMP is most probably the second messenger for β -adrenoceptor action on myometrial tissues, because dibutyl-cyclic AMP and forskolin, which permeates into the cell interior and activates the catalytic unit of adenylate cyclase, are both capable of causing an inhibitory effect similar to isoprenaline (for review see Bülbring & Tomita, 1987; Marshall & Fain, 1984; Maruta & Osa, 1989). Furthermore, it was recently shown that relaxin increased the cellular content of cyclic AMP in the rat myometrial cells in culture (Hsu *et al.*, 1985). In the present experiments, the cyclic AMP content of the longitudinal myometrium of the oestrogen-primed rat was increased to 186% of the control by the application of 150 mu relaxin in Krebs solution containing 0.6 mM Mg, and the dose of the agent depressed the phasic contraction to 16% of the control response (Table 1). Therefore, cyclic AMP may in some way participate in the inhibitory effect of relaxin.

It has been reported that the external presence of Mg and other divalent cations enhances the physiological effects of several agents such as vasopressin and oxytocin (vascular

Figure 11 Effects of pretreatment with 0.6 mM Mg (a) and 0.6 mM Mn (b) on the cyclic AMP content increased by 10^{-9} M isoprenaline (Δ , $\blacktriangle$), 10^{-7} M forskolin ($\square$, $\blacksquare$) and 100 mu relaxin ($\circ$, $\bullet$). Open and filled symbols show cyclic AMP content measured without and with pretreatment with Mg or Mn. The cyclic AMP content is expressed as a % of the content measured 120 min after incubation with Mg-free solution for other muscle strips taken from the same animal. The application of Mg or Mn, and agents is indicated by bars and broken lines. Figures in parentheses indicate the number of measurements, and vertical bars indicate s.e.mean. See text for further explanation.

muscle, Somlyo *et al.*, 1966; myometrium, Schild, 1969). One action was shown to be due to the enhancement of binding between the agonist and receptor (Soloff & Grzonka, 1986). On the other hand, the enhancement of β -adrenoceptor inhibition by Mg application via an intracellular process has been proposed in some smooth muscle tissues (myometrium, Maruta & Osa, 1989; taenia, Maruta *et al.*, 1989). In the present experiments, the intracellular cyclic AMP content was more elevated by administration of β -agonists and relaxin measured in the Mg-free solution, after brief application of 0.6 mM Mg, where the experimental design eliminated the facilitatory effect of Mg on the binding of relaxin to the surface membrane receptors (Mercado-Simmen *et al.*, 1980).

A question that arises is what happens during pretreatment with divalent cations. Free $[Mg]_i$ in immature rat uterus was estimated to be between 200 and 400 μM , and increased by 25% at 24 and 48 h after intraperitoneal injection of 17 β -oestradiol (Degani *et al.*, 1984). A recent report (Nakayama & Tomita, 1990) has shown that intracellular free Mg concentration ($[Mg]_i$) of guinea-pig taenia caeci is estimated to be 310 μM , and can be changed by altering the extracellular ionic environment. The $[Mg]_i$ was not altered by removal of external Mg, but decreased to 8.3 μM in 100 min when both Mg and Ca were omitted. The $[Mg]_i$ recovers completely in about 25 min on readmission of 1.2 mM Mg. On the other hand, Mn ion has long been known as an inorganic Ca antagonist (for reviews see Bolton, 1979; Weiss, 1981). Yet its action on contraction is complicated, and is not fully understood; $MnCl_2$ modifies the Ca influx and penetrates the cell, thus causing either a suppression (Keene *et al.*, 1972) or an acceleration (Shibata, 1969; Osa, 1974; Sakai & Uchida, 1981; Itoh *et al.*, 1982; Lategan & Brading, 1988) of the mechanical responses in various visceral muscles. Intracellular accumulation of Mn was experimentally proved to occur in rat ventricular muscle, in which the baseline tension was gradually elevated when exposed to 0.2 mM Mn (Chapman & Ellis, 1977). Thus, if a similar situation is applicable to myometrium, it can be assumed that the cells are loaded with Mg or Mn by pretreatment with the respective ions, and these are not removed.

In intact cells and cell-free systems, it has been reported that the adenylate cyclase activity linked to β -adrenoceptor activation is facilitated by adding Mg or Mn (Krall *et al.*, 1988; Mittag *et al.*, 1988) or can be directly activated by these ions (Braun *et al.*, 1972; Neer, 1979; Cech *et al.*, 1980; Cech & Maguire, 1982; Mittag *et al.*, 1988; Arkinstall & Jones, 1990). Effects of Mg and Mn could be in the same or the opposite direction in regulating adenylate cyclase activity depending on the agonists and tissues (Limbird *et al.*, 1979; Cech *et al.*, 1980; Cech & Maguire, 1982). As far as the present results are concerned, 'intracellular' Mg and Mn share in common the enhancing effect by themselves on the cyclic AMP production (Figure 11). The relationship between cyclic AMP production and contractile depression is linear in the case of forskolin application such that cyclic AMP was more increased and contraction was more strongly depressed when the muscle was pretreated with either Mg or Mn. Therefore, the action site of divalent cations is probably the catalytic unit of adenylate cyclase. The same idea could also be applied to the case of relaxin action after the pretreatment with Mg. On the other hand, cyclic AMP production in response to 10^{-9} M isoprenaline was not significantly changed by pretreatment with either Mg or Mn, while the contractile depression was strongly enhanced (Figure 10; Maruta & Osa, 1989). Furthermore, the cyclic AMP production by relaxin was not significantly altered with or without Mn pretreatment, whereas the contractile depression became much smaller after Mn pretreat-

ment. These are the experimental findings suggesting that the cellular content of cyclic AMP does not parallel the contractile depression. In general, there are several sequential interactions from the activation of a receptor by a hormone to the final effect, and the interactions take place in the membrane as well as the intracellular phase involving myosin kinase (for review see Adelstein, 1980). H-8, a potent protein kinase A inhibitor, effectively reversed the attenuation by both isoprenaline and relaxin of the oxytocin-induced increase in $[Ca]_i$ and phosphoinositide turnover of myometrial cells in culture (Anwer *et al.*, 1990). At present, therefore, it seems possible that Mn blocks or potentiates some step(s), e.g. protein kinase(s), so that contractile depression was changed in different ways in response to relaxin or isoprenaline, respectively.

Investigations of the contractile response revealed some unexpected features of the action of relaxin especially concerning the effects of divalent cations; the depression of phasic contractions persisted after the removal of relaxin, when Mg was present in the recovery solution, and raising the external Ca to 7.5 mM did not restore the amplitude of phasic contraction. During the persistent depression, the removal of external Mg was effective in restoring the phasic contraction. Therefore, the enhancement of stabilizing action of Mg at the membrane to prevent Ca-influx is suggested. Assuming that the increase in $[Mg]_i$ enhances the relaxin action and *vice versa*, it may be deduced that $[Mg]_i$ was reduced when the tissue was cooled in Mg free solution and hence the depressive action of relaxin on contraction became reduced.

As frequently reported by previous investigators, hyperpolarization of the membrane and reduction of membrane resistance are the electrophysiological manifestation of stimulation of β -adrenoceptors in the myometrial membrane, suggesting an increase in K-conductance (for review see Bülbring & Tomita, 1987). On the other hand, Chamley & Parkinson (1984) showed that the plateau potential in the myometrium of the pregnant and oestrogen-treated rat is specifically depressed by relaxin added to the Krebs solution, and the resting membrane was not hyperpolarized by relaxin up to 300 mV. This observation was confirmed on the longitudinal myometrium (3E) in the present experiments. It is furthermore observed that the depression of contraction by 50 mV relaxin is probably caused secondarily to the shortening of the action potential, whereas the contractile depression was caused by 150 mV relaxin in spite of no change in action potential. For the latter finding, inhibitory effects of relaxin on the contractile machinery bypassing the membrane response should be taken into account. It has been proposed that a cyclic AMP-mediated phosphorylation of myosin light chain kinase decreases its affinity for calmodulin (Conti & Adelstein, 1981; Higashi *et al.*, 1983), whereby relaxin relaxes the myometrium (Nishikori *et al.*, 1983; Hsu & Sanborn, 1986).

In conclusion, the present physiological results are compatible with the view that cyclic AMP acts as a second messenger of relaxin in depressing the myometrial contraction. However, it is possible that intracellular signal transduction systems are different between β -adrenoceptor-mediated and relaxin actions.

We are grateful to the National Hormone and Pituitary Program, U.S.A., for supplying porcine relaxin, to Prof. T. Tsukamoto, Saga University, for informing us of the revised technique of measuring cyclic AMP, to the staff of Biochemistry, Yamaguchi University, for the use of their facilities, and to Dr K.E. Creed, Murdoch University, for critical reading of the manuscript. This work was partly supported by the Grant in Aid of Scientific Research from the Ministry of Education of Japan (Project number 02670039).

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(Received May 29, 1991)

Revised July 7, 1991

Accepted July 24, 1991)