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The lack of a role for potassium channel opening in the action of relaxin in the rat isolated uterus; a comparison with levcromakalim and salbutamol

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1 The effects of relaxin *in vitro* in the isolated uterus from the non-pregnant rat were compared with those of levcromakalim and salbutamol in tissue bath, $^{42}K^+$-efflux and electrophysiological studies, to determine whether relaxin exhibits the characteristics of an opener of K_{ATP}-channels.

2 In uterus exposed to oxytocin (0.2 nM), tetraethylammonium (TEA, 10 mM) and glibenclamide (10 μM) produced large rightward shifts of the $\log_{10}$ concentration-effect curve to levcromakalim (125 fold and 118 fold, respectively). TEA (10 mM) caused only small rightward shifts of the $\log_{10}$ concentration-effect curves to salbutamol and relaxin (5.2 fold and 7.5 fold respectively). Glibenclamide did not antagonize salbutamol or relaxin.

3 Levcromakalim (0.2 and 2 μM) suppressed the spasm evoked by low ($\leqslant$40 mM) but not high ($>$40 mM) concentrations of KCl. Salbutamol (1.5 nM) inhibited the spasm evoked by low concentrations of KCl ($\leqslant$40 mM). Salbutamol (15 nM) and relaxin (3 and 30 nM) inhibited the spasm evoked by low and high concentrations of KCl (10–80 mM).

4 Relaxin (0.12 μM) did not produce an increase in $^{42}K^+$-efflux from longitudinal segments of rat myometrium. Exposure of tissues to relaxin (0.12 μM), in the presence of diltiazem (1 μM) plus KCl (20 mM), resulted in a small increase in $^{42}K^+$-efflux of short duration.

5 Electrophysiological recording showed that the phasic spasms of the uterus exposed to oxytocin (0.2 nM) were accompanied by bursts of spiking activity superimposed upon a plateau potential. Inhibition of the mechanical activity of the uterus by levcromakalim (2 and 10 μM), salbutamol (30 nM) or relaxin (0.18 μM) was accompanied by a reduction in the duration of the plateau potential and the number of spikes without membrane hyperpolarization.

6 Unlike levcromakalim, relaxin did not selectively inhibit the spasm evoked by low concentrations of KCl and was not markedly antagonized by TEA or glibenclamide. Under conditions where a cromakalim-induced increase of the $^{42}K^+$-efflux rate has been demonstrated, relaxin had only a very small effect. In isolated uterus from the rat, in contrast to observations *in vivo*, relaxin did not exhibit the characteristics of an opener of K_{ATP}-channels suggesting that another mechanism accounts for its inhibitory action.

Keywords: Uterus; relaxin; levcromakalim; salbutamol; K-channels; tetraethylammonium; glibenclamide; $^{42}K^+$-efflux; electrophysiology

Introduction

Relaxin is a polypeptide hormone which selectively, potently and reversibly inhibits uterine contractions both *in vivo* and *in vitro* (Downing & Hollingsworth, 1993; Sherwood, 1994). The inhibitory effect occurs within seconds of administration suggesting that relaxin interacts directly with a plasma membrane receptor and activates mechanisms which result in enhanced cellular enzyme activity and/or ion channel modulation. The signal transduction pathway that is activated following relaxin-receptor interaction is not clearly defined, although much attention has focused on the possible involvement of an adenosine 3′:5′-cyclic monophosphate (cyclic AMP)-dependent pathway (Sanborn *et al.*, 1994).

Application of relaxin to isolated uterine strips in tissue baths or myometrial cells in culture elicited time- and concentration-dependent rises in cyclic AMP concentrations which followed rather than preceded the mechanical inhibition (Judson *et al.*, 1980; Sanborn *et al.*, 1980). Also, *in vivo* administration of relaxin resulted in only a small rise in uterine cyclic AMP concentrations of short duration which did not correlate with the prolonged inhibition of myometrial activity (Downing *et al.*, 1992). These data indicate that a rise in the cellular cyclic AMP concentration is not the causal mechanism involved in the inhibitory action of relaxin in myometrium.

Hughes & Hollingsworth (1995) suggested that the primary action of relaxin involves modification of plasma membrane ion fluxes rather than a receptor coupled via a G-protein to adenylyl cyclase and cyclic AMP generation. St-Louis (1981) demonstrated that relaxin was able to inhibit the spasm evoked by 20 mM KCl but not that evoked by 30 mM KCl in rat isolated uterus. Such a profile of action is seen with drugs that stimulate the opening of potassium (K) channels, for example with levcromakalim, the purported opener of ATP-sensitive K (K_{ATP})-channels (Edwards & Weston, 1994). Glibenclamide, an inhibitor of K_{ATP}-channels, has been shown to antagonize selectively levcromakalim, or its racemate cromakalim, in a number of smooth muscles (Edwards & Weston, 1994) including rat uterus, both *in vivo* and *in vitro* (Piper *et al.*, 1990; Downing & Hollingsworth, 1991). Glibenclamide also selectively antagonized relaxin *in vivo* causing a non-parallel, rightward shift of its concentration-effect curve but it did not antagonize salbutamol (Downing & Hollingsworth, 1991). This observation, together with that of St-Louis (1981), led us to the hypothesis that relaxin may share a common mechanism

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of action with cromakalim, namely the opening of K_{ATP}-channels in the plasma membrane (Downing & Hollingsworth, 1991; 1993). K-channel opening drugs classically stimulate efflux of $^{42}K^+$, or its marker $^{86}Rb^+$, and produce a marked hyperpolarization of the plasma membrane in a variety of isolated tissues (Edwards & Weston, 1994). Such changes have been difficult to demonstrate in uterus from the non-pregnant or pregnant rat (Hollingsworth *et al.*, 1987; 1989) but cromakalim-induced $^{42}K^+$ efflux can be seen in depolarizing conditions (Piper & Hollingsworth, 1995).

The aim of this study was to investigate more fully whether the inhibitory action of relaxin on the rat isolated uterus involves K-channel opening, by determining whether relaxin exhibits the characteristics of an opener of K-channels in general or more specifically K_{ATP}-channels. The hypothesis was tested using tissue bath, $^{42}K^+$-efflux and electrophysiological experiments. The effects of relaxin were compared with those produced by levcromakalim (Edwards & Weston, 1994) and salbutamol, a β_2-adrenoceptor agonist (Diamond, 1990). Preliminary results have been presented to the British Pharmacological Society (Hughes *et al.*, 1992).

Methods

Female, non-pregnant Sprague-Dawley rats (200–300 g) were purchased from Charles River Ltd. Rats were treated 18–24 h prior to experimentation with 17β-oestradiol benzoate (100 μg kg^{-1}, s.c.).

Tissue bath experiments

Rats were killed and uterine horns removed and placed in a physiological salt solution (PSS). Uterine segments were set up according to the method of Hughes & Hollingsworth (1995). Mechanical responses were measured as the integral of the tension versus time curve by the method of Granger *et al.* (1985).

Effects of relaxants against spasm evoked by oxytocin and antagonism by potassium channel inhibitors Following equilibration, tissue segments were incubated for 20 min in PSS containing either tetraethylammonium (TEA, 10 mM) or glibenclamide (10 μM) (test tissues) or the relevant vehicle (control tissues). Subsequently the segments were incubated for 15 min in PSS containing oxytocin (0.2 nM) plus the antagonist/vehicle (total exposure to antagonist/vehicle 35 min). The segments were exposed to oxytocin (0.2 nM) for 15 min to allow regular and maximal phasic spasms to develop before relaxant (levcromakalim, salbutamol or relaxin) was added to the tissue bath in the continued presence of the spasmogen. A single concentration-effect curve for a relaxant was constructed with each tissue segment. Two fold increments in relaxant concentration were applied at 10 min intervals in a cumulative manner until 100% or maximal inhibition was attained.

In each segment of tissue the % spasm remaining was determined by expressing the integral of the tension recorded from 5 to 10 min after addition of each concentration of relaxant as a percentage of the integral of the tension recorded in the 5 min period prior to the administration of any relaxant. pD_2 values were calculated by the method of Foster & Hollingsworth (1985). The pD_2 values were averaged for each experimental group to give the mean $pD_2 \pm$ s.e.mean. The difference in potencies of the relaxants against oxytocin (0.2 nM)-induced tension in the presence and absence of the K-channel inhibitor were calculated as the difference of the corresponding mean $pD_2 \pm$ s.e.mean values or $\log_{10}$ concentration ratio ($\log_{10}$ CR) $\pm$ s.e.mean. For each relaxant concentration the mean $\pm$ s.e.mean % spasm remaining are presented graphically.

Effects of relaxants against spasm evoked by KCl Following equilibration in PSS, three cumulative concentration-effect curves were constructed to KCl (10, 20, 40, 80 mM) at hourly intervals. The concentration of KCl refers to the KCl added to the bath and does not include the concentration of K^+ present in the PSS. Subsequently, tissues were incubated in PSS containing either relaxant (levcromakalim 0.2 or 2.0 μM, salbutamol 1.5 or 15 nM, relaxin 3 or 30 nM) or the relevant vehicle for 30 min before and during the construction of a third concentration-effect curve for KCl. The relaxant concentrations chosen correspond to approximately 1 fold and 10 fold the pD_2 values of the relaxants versus the spasm induced by oxytocin (0.2 nM).

Data from the 1st curve were disregarded since the size of the spasm elicited by each concentration of KCl was larger in the 1st curve than in the 2nd and 3rd curves. The integral of the tension vs time curve for the period from 5 to 10 min after each addition of KCl was expressed as a proportion (%) of the maximum integral recorded in the 2nd curve to give the spasm as % maximum. The effect of relaxant/vehicle upon the 3rd KCl curve was assessed by comparing the % maximum spasm in the 3rd curve (test tissues – relaxant-exposed; control tissues – vehicle-exposed) with that in the combined control curve at corresponding concentrations of KCl.

Efflux of $^{42}K^+$

Cox (1990) demonstrated in rat tail artery that the cromakalim-induced $^{42}K^+$ efflux was larger when carried out in PSS containing raised K^+ concentrations than that seen in normal PSS. It has been shown that in the presence of the calcium channel blocker diltiazem (1 μM) and KCl (20 mM) the cromakalim-induced $^{42}K^+$ efflux in rat uterus was larger than that seen in normal PSS (Piper & Hollingsworth, 1995). The effects of relaxin were investigated, therefore, in the presence of diltiazem (1 μM) plus KCl (20 mM) as well as in normal PSS.

The technique of Piper & Hollingsworth (1995) was followed to measure $^{42}K^+$ efflux. Briefly, longitudinal myometrial strips were loaded with $^{42}K^+$ by incubation in PSS containing $^{42}K_2CO_3$ (1.35 μCi ml^{-1}) for 3 h. After loading, the efflux of $^{42}K^+$ from the uterine segments was assessed at 4 min intervals (except the first period which was 8 min long). For each group of tissues the first three sets of tubes (0–16 min) were discarded as the periods during which surface radioactivity was washed off the tissue. The next three periods (16–28 min) represent the basal efflux in normal PSS. For the next six periods (28–52 min) each group of tissues was exposed to PSS plus various drugs: group (i) saline; group (ii) oxytocin (20 nM); group (iii) relaxin (0.12 μM) and group (iv) KCl (20 mM). Two groups of tissues were exposed to a combination of drugs. Group (v) tissues were exposed to PSS containing diltiazem (1 μM) for 28 min (i.e. 0–28 min) and then to diltiazem (1 μM) plus KCl (20 mM) for 24 min (28–52 min). Group (vi) tissues were exposed to diltiazem (1 μM) for 20 min (0–20 min), then to diltiazem (1 μM) plus KCl (20 mM) for 8 min (20–28 min) and then to diltiazem (1 μM) plus KCl (20 mM) plus relaxin (0.12 μM) for 24 min (28–52 min). All tubes contained PSS only for the last two periods (52–60 min). The $^{42}K^+$ content of strips was ascertained and corrected for background count and half-life decay (12.4 h). Data were expressed as the efflux rate coefficient (fractional loss of $^{42}K^+$ from the tissue per min) expressed as a percentage. The mean efflux rate coefficient $\pm$ s.e.mean of $n = 8$ tissues was calculated at the mid-point of each period.

Analyses of drug effects on efflux rate coefficients were performed by 2-way analysis of variance (drug and time), followed by Student's unpaired t test for comparison of means from different groups at corresponding time points. The effect of relaxin (0.12 μM) upon the efflux rate of $^{42}K^+$, in the presence of diltiazem (1 μM) and KCl (20 mM; 28–52 min), was compared to that occurring in the presence of diltiazem (1 μM) plus KCl (20 mM) alone (24–28 min) in the same tissues using Student's paired t test.

Electrophysiology experiments

Each uterine horn was mounted on a perspex tissue holder designed for the simultaneous recording of the intracellular electrical activity and the mechanical activity of a contiguous segment of tissue (Small & Weston, 1980). Tissues were equilibrated in normal PSS at 37°C for 30–60 min and then in PSS containing oxytocin (0.2 nM) for 30 min before recording commenced.

Glass microelectrodes (resistance 40–120 MΩ) were filled with 3 M KCl and mounted on a flexible silver wire. Following the impalement of a uterine cell and stabilization of the recording, a control period of 2–4 spasms and associated electrical activity was recorded. Drug effects were recorded for at least 4 min. The effects of levcromakalim (2 μM, 10 μM), salbutamol (15 nM) and relaxin (0.18 μM) were investigated. These concentrations correspond to concentrations which were maximally effective against oxytocin (0.2 nM)-induced spasms in tissue bath studies.

The electrical activity associated with the phasic mechanical activity induced by oxytocin (0.2 nM) consisted of a burst of spike potentials superimposed upon a plateau potential. Measurements made during the period prior to drug addition were pooled for all of the impalements made ($n=30$), regardless of which relaxant was subsequently added, to give the mean control data. Measurements made following the addition of relaxant were: the inter-spasm and inter-burst periods which refer to the length of time between the last two recorded spasms or bursts of electrical activity prior to complete inhibition; the amplitude and duration of spasms, plateaus and spiking activities which refer to the parameters of the last spasm or burst of electrical activity prior to complete inhibition and the membrane potential which refers to that measured following complete inhibition of mechanical activity.

Drugs and solutions

The following drugs were used: levcromakalim (SmithKline Beecham); salbutamol sulphate (Glaxo); glibenclamide (Hoescht); diltiazem (Synthelabo); tetraethylammonium, oxytocin acetate and 17β-oestradiol benzoate (Sigma); KCl (BDH Chemicals); $^{42}K_2CO_3$ (Risley Reactor, University of Manchester). Porcine relaxin was isolated and purified from pregnant sow ovaries by the method of Sherwood & O'Byrne (1974) by Dr S.J. Downing. Relaxin was bioassayed *in vitro* by inhibition of the electrically-stimulated uterus from oestrogen-treated rats (100 μg kg^{-1}), against highly purified porcine relaxin, kindly donated by Dr O.D. Sherwood, as standard. Our relaxin preparation was found to be equipotent with highly purified relaxin. Stock solutions were prepared in the following solvents: levcromakalim, 70% v/v ethanol; salbutamol, 0.1 M HCl; glibenclamide, 95% v/v ethanol; diltiazem, TEA, oxytocin acetate, KCl, twice distilled water; oestradiol benzoate, arachis oil; relaxin, saline. Serial dilutions of the stock dilutions were made in saline on the day of use.

The normal PSS had the following composition (mM): Na^+ 143.0, K^+ 5.9, Mg^{2+} 1.2, Ca^{2+} 2.6, $H_2PO_4^-$ 1.2, SO_4^{2-} 1.2, Cl^- 128.0, HCO_3^- 25.0 and glucose 11.0. The PSS used in the efflux experiments was prepared by replacing the KCl and KH_2PO_4 with $^{42}K_2CO_3$.

Analysis of data

Data are expressed as means ± s.e.mean. In tissue bath and electrophysiological experiments comparison of means was performed by Student's two-tailed, unpaired t test with significance identified at $P<0.05$.

Results

Tissue bath

Effects of relaxants against spasm evoked by oxytocin (0.2 nM) and antagonism by potassium channel inhibitors Cumulative administration of levcromakalim (0.025–1.6 μM), salbutamol (0.025–16 nM) or relaxin (0.8–25 nM) produced concentration-dependent reduction of both the amplitude and frequency of oxytocin (0.2 nM)-induced spasms, effects which were reversed on washing in oxytocin (0.2 nM)-containing PSS. The pD_2 values are shown in Table 1.

TEA (10 mM) increased the amplitude of the oxytocin (0.2 nM)-induced phasic spasms but glibenclamide (10 μM) had no effect. TEA (10 mM) and glibenclamide (10 μM) each caused a large rightward shift of the $\log_{10}$ concentration-effect curve for levcromakalim (125 fold and 118 fold respectively; Table 1). TEA (10 mM) caused small rightward shifts of the $\log_{10}$ concentration-effect curves for salbutamol and relaxin (5.2 fold and 7.5 fold respectively; Table 1). Glibenclamide (10 μM) had no effect on the position of the $\log_{10}$ concentration-effect curves for salbutamol or relaxin (Table 1).

Effects of relaxants against the spasm evoked by KCl Levcromakalim (0.2 and 2.0 μM) reduced the size of the spasm evoked by 10, 20 and 40 mM KCl but had no inhibitory effect upon the spasm evoked by 80 mM KCl (Figure 1a(i)). Salbutamol (1.5 nM) reduced the size of the spasm evoked by 10, 20 and 40 mM KCl (Figure 1b(i)). A higher concentration of salbutamol (15 nM) caused a greater inhibition of the spasm evoked by the lower concentrations of KCl and reduced the size of the spasm evoked by 80 mM KCl (Figure 1b(i)). Relaxin (3 and 30 nM) reduced the size of the spasm evoked by all concentrations of KCl, the degree of inhibition was larger with the higher concentration of relaxin (Figure 1c(i)). In control experiments, the position and shape of the KCl concentration-effect curve was unaffected by time or the vehicle for the relaxants (Figure 1a(ii),b(ii),c(ii)).

Efflux of $^{42}K^+$

Effect of oxytocin and relaxin on $^{42}K^+$ efflux in normal PSS The mean basal rate of efflux of $^{42}K^+$ ranged between 1.38 and 1.58 % min^{-1} (Figures 2 and 3). On first exposure to saline, the vehicle for relaxin, there was a small increase in the efflux rate compared to pre-saline values in the same tissues, which was probably artifactual (Figure 2). The rate of efflux

Table 1 The effects of TEA (10 mM) and glibenclamide (10 μM) against the inhibitory actions of levcromakalim, salbutamol and relaxin versus oxytocin (0.2 nM)-induced spasm in isolated uterus of the non-pregnant rat

	Control	*+TEA*		*Control*	*+Glibenclamide*	
Agonist	pD_2	pD_2	*mean CR*	pD_2	pD_2	*mean CR*
Levcromakalim	6.56 ± 0.15	4.46 ± 0.13**	125	6.81 ± 0.27	4.74 ± 0.26*	118
Salbutamol	9.19 ± 0.02	8.46 ± 0.09**	5.2	8.80 ± 0.08	8.85 ± 0.08	0.9
Relaxin	8.68 ± 0.07	7.80 ± 0.37*	7.5	8.42 ± 0.12	8.13 ± 0.13	1.9

pD_2 values are means ± s.e.mean, $n=5-8$. Mean CR = represents the difference in potency of the relaxant in the presence and in the absence of the K^+-channel inhibitor. *$P<0.05$, **$P<0.01$ indicate a significant difference from the corresponding control pD_2 value.

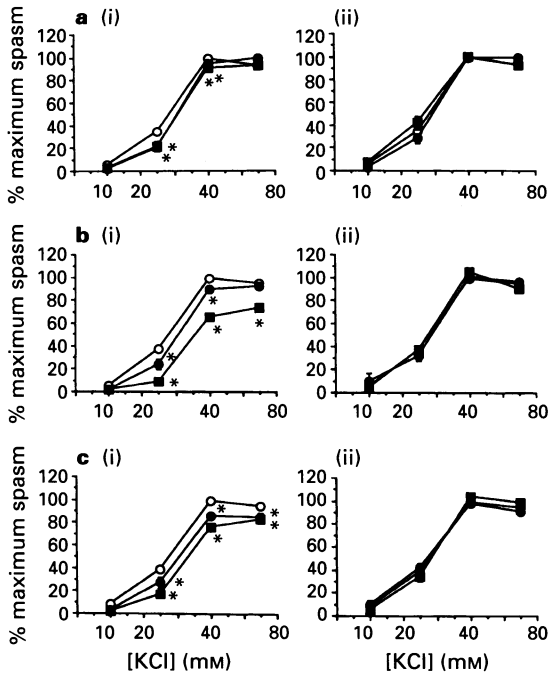

Figure 1 Effects of relaxants against spasm evoked by KCl in the isolated uterus from the 17β-oestradiol benzoate-treated, non-pregnant rat. Effects are shown in the absence (○) and in the presence of (a(i)) levcromakalim (0.2 μM, ●; 2.0 μM, ■), (b(i)) salbutamol (1.5 nM, ●; 15 nM, ■) or (c(i)) relaxin (3 nM, ●; 30 nM, ■). Respective time-matched, vehicle-treated controls are shown in (a(ii)), (b(ii)) and (c(ii)). Points indicate the means ± s.e.mean ($n = 7-32$). * Represents a significant ($P < 0.05$) reduction in the size of the spasm compared to that in the initial curve at the corresponding concentration of KCl.

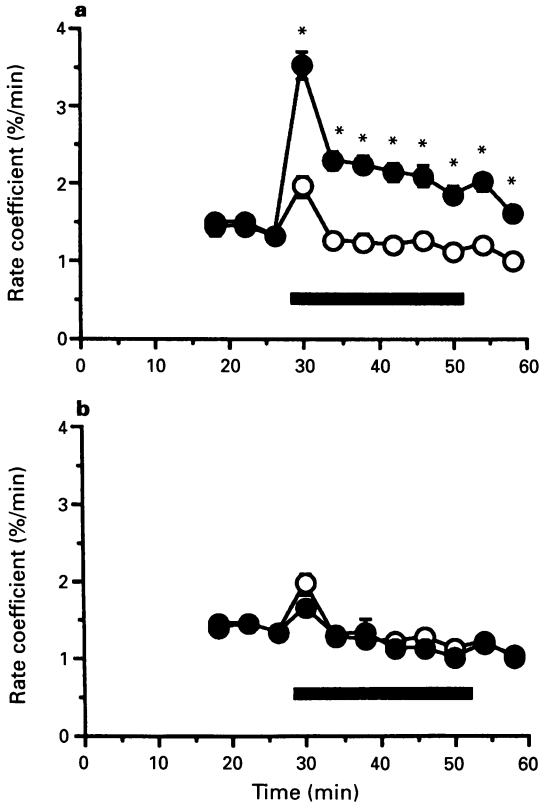

Figure 2 The effect of (a) oxytocin (20 nM, ●) and (b) relaxin (0.12 μM, ●) on $^{42}K^+$-efflux from the longitudinal myometrium from the 17β-oestradiol benzoate-treated, non-pregnant rat. Also shown are the effects of saline (○), the vehicle for relaxin. Tissues were exposed to saline, oxytocin or relaxin for the period 28–52 min (as represented by the horizontal bar). * Represents a significant ($P < 0.01$) increase of the test value from the corresponding value in saline-exposed tissues at the same time point. Points indicate the means ± s.e.mean ($n = 7-8$).

was constant for the remainder of the experiment. In other segments of tissue, upon exposure to oxytocin (20 nM) the efflux rate was much greater than that in saline-exposed tissues (Figure 2a). In a further set of tissues relaxin (0.12 μM) had no stimulatory effect upon the rate of $^{42}K^+$ efflux relative to that measured in the presence of saline (Figure 2b).

Effect of relaxin on $^{42}K^+$ efflux in depolarizing conditions The effects of raising the K^+ concentration and adding diltiazem (1 μM) upon the rate of $^{42}K^+$ efflux from the tissues are collated in Figure 3. Exposure of tissues to KCl (20 mM) raised the efflux rate from a mean basal level of approximately 1.58% min^{-1} to around 3.0% min^{-1} (Figure 3a). Diltiazem did not alter the basal rate of $^{42}K^+$ efflux but reduced slightly the efflux due to KCl (20 mM) (Figure 3a). During the first 2 periods of exposure to relaxin (0.12 μM) there was a very small stimulation of the $^{42}K^+$ efflux rate above that measured in the presence of KCl and diltiazem alone in the same tissues. In subsequent collection periods the $^{42}K^+$ efflux rate in relaxin-exposed tissues was not different from that recorded in the presence of KCl (20 mM) plus diltiazem (1 μM) (Figure 3b).

Electrophysiological experiments

Intracellular electrophysiological recording from the uterus Oxytocin (0.2 nM) induced phasic spasms every 37.8 ± 2.1 s which were of 8.1 ± 0.6 g tension and lasted for 6.8 ± 0.2 s ($n = 30$). The electrical activity associated with these spasms consisted of a plateau potential (amplitude 10.6 ± 0.4 mV, duration 7.1 ± 0.2 s) upon which spike potentials, 39.8 ± 0.8 mV in amplitude, were superimposed. Generally 5–6 discrete spike potentials were seen on top of each plateau potential. Between the bursts of electrical activity, approximately every 37.7 ± 2.1 s, the membrane potential approached a resting value of −63.3 ± 0.9 mV ($n = 30$). Prior to the firing of each burst of electrical activity a slowly developing depolarization or prepotential was usually observed. The mechanical and electrical activities were extremely well synchronized.

Effects of levcromakalim In 3 out of 5 tissues levcromakalim (2 μM) caused 100% inhibition of both the mechanical and electrical activities within 4–6 min. In these 3 tissues the duration of the last spasm recorded prior to complete inhibition was reduced (levcromakalim 3.6 ± 0.6 s; control 6.8 ± 0.2 s). These spasms were associated with a reduced frequency of spiking activity (levcromakalim 3.0 ± 0.6 spikes/burst; control 5.7 ± 0.4 spikes/burst). In all other respects the mechanical and electrical activities were not modified from the pre-drug control activities. The resting membrane potential recorded following 100% inhibition was also unchanged from control values (levcromakalim −62.7 ± 6.6 mV, control −63.3 ± 0.9 mV). In the other two tissues only partial inhibition of the mechanical and electrical activities was seen after 8 min. The resting membrane potential between bursts was not modified.

Levcromakalim (10 μM) induced complete inhibition of the mechanical and electrical activities within 3 min in all tissues ($n = 8$) which was abrupt in nature (Figure 4). These spasms and their associated electrical activities were not modified from those recorded prior to drug administration. The resting membrane potential recorded following complete inhibition of mechanical and electrical activities was not different from control values (levcromakalim −64.8 ± 2.9 mV, control −63.3 ± 0.9 mV).

Effect of salbutamol Salbutamol (30 nM) caused a gradual decline of the mechanical activity, 3–6 spasms were recorded over approximately 6 min prior to complete inhibition ($n=9$). The time between spasms was not altered (salbutamol 46.6±4.1 s, control 37.8±2.1 s). The last spasm recorded before complete inhibition was reduced in amplitude (salbutamol 4.2±1.2 g, control 8.1±0.6 g) and duration (salbutamol 5.1±0.4 s, control 6.8±0.2 s). In 6 of the 9 tissues, inhibition of the mechanical activity was associated with a parallel inhibition of the electrical activity. In these 6 tissues the time between the last two bursts of electrical activity was lengthened by approximately 30% (salbutamol 48.8±4.4 s, control 37.7±2.1 s). The duration of the plateau potential was reduced (salbutamol 5.0±0.3 s; control 7.1±0.2 s). The spiking activity associated with the last recorded spasm was reduced in amplitude (salbutamol 34.2±2.0 mV; control 39.8±0.8 mV) and frequency (salbutamol 4.0±0.3 spikes/burst, control 5.7±0.4 spikes/burst) compared to pre-drug spiking activity. The resting membrane potential recorded following complete inhibition was unchanged (salbutamol −65.0±2.1 mV, control −63.3±0.9 mV). In the other 3 tissues bursts of electrical activity were still evident following complete inhibition of the mechanical activity. These bursts of ongoing activity had a plateau potential of shorter duration, spiking activity was reduced in frequency and some single action potentials were evident.

Effect of relaxin Relaxin (0.18 μM, $n=8$) produced less inhibition of the mechanical activity in the electrophysiological system than in the tissue bath system. After 8–10 min the amplitude of the spasm was reduced by only 24±7% (relaxin 6.2±0.4 g, control 8.1±0.6 g) and the duration of the spasm by 29±5% (relaxin 4.8±0.3 s, control 6.8±0.2 s) (Figure 5). The length of time between spasms was increased by 37% (relaxin 51.8±6.4 s, control 37.8±2.1 s) and that between bursts of electrical activity by 38% (relaxin 52.1±5.5 s; control 37.7±2.1 s). The electrical activity associated with these partially inhibited spasms was not modified in terms of spike amplitude and duration. The spiking activity on top of the plateau was reduced (relaxin 3.9±0.8 spikes/burst, control 5.7±0.4 spikes/burst). The resting membrane potential was unchanged (relaxin −61.5±3.8 mV, control −63.3±0.9 mV).

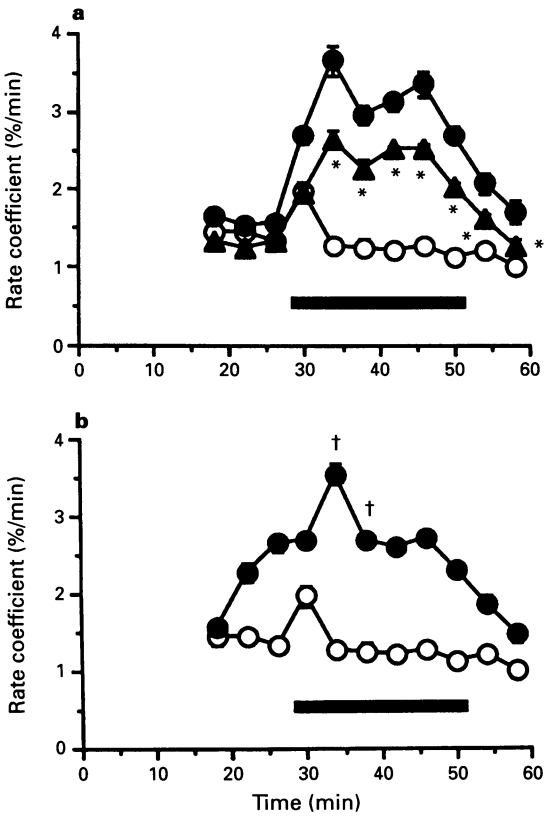

Figure 3 The effect of (a) KCl and diltiazem and (b) relaxin plus KCl and diltiazem on $^{42}K^+$-efflux from longitudinal myometrium from the 17β-oestradiol benzoate-treated, non-pregnant rat. (a) Tissues were exposed to saline (○) for the period 28–52 min, to KCl (20 mM, ●) for the period 28–52 min or to diltiazem (1 μM) alone for the period 0–28 min and then to diltiazem (1 μM) plus KCl (20 mM, ▲) for the period 28–52 min. * Represents a significant ($P<0.01$) decrease in the efflux rate in diltiazem plus KCl-exposed tissues (▲) compared to that in KCl-exposed tissues (●) at the same time point. (b) Tissues were exposed to saline (○) for the period 28–52 min, or to diltiazem (1 μM) alone for the period 0–20 min, then to diltiazem (1 μM) plus KCl (20 mM) for the period 20–28 min and finally to diltiazem (1 μM) plus KCl (20 mM) plus relaxin (0.18 μM; ●) for the period 28–52 min. The horizontal bar represents the period of exposure to relaxin. †Represents a significant ($P<0.01$) increase of the efflux rate during the period of exposure to diltiazem plus KCl plus relaxin compared to that during exposure to diltiazem plus KCl. Points indicate the means±s.e.mean ($n=7-8$).

Discussion

This study compared the characteristics of relaxin in uterine tissue in tissue bath, $^{42}K^+$-efflux and electrophysiological studies with those of levcromakalim and salbutamol.

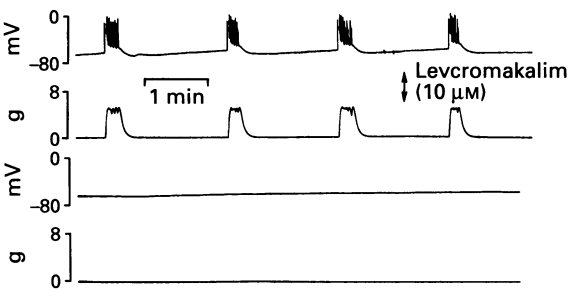

Figure 4 The effect of levcromakalim (10 μM) on the electrical and mechanical activities induced by oxytocin (0.2 nM) in the isolated uterus from the 17β-oestradiol benzoate-treated, non-pregnant rat. The upper trace represents the membrane potential changes (mV) and the lower trace represents the tension changes (g). Activity was recorded before and after the addition of levcromakalim (indicated by the arrow). The lower two traces are contiguous with the upper two traces.

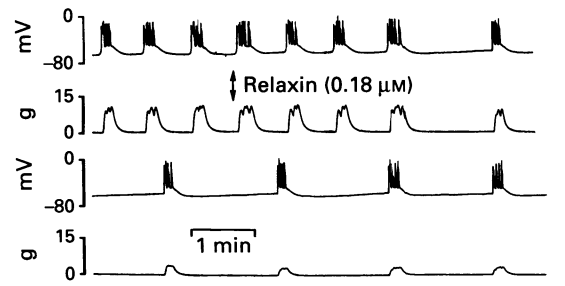

Figure 5 The effect of relaxin (0.18 μM) on the electrical and mechanical activities induced by oxytocin (0.2 nM) in the isolated uterus from the 17β-oestradiol benzoate-treated, non-pregnant rat. The upper trace represents the membrane potential changes (mV) and the lower trace represents the tension changes (g). Activity was recorded before and after the addition of relaxin (indicated by the arrow). The lower two traces are contiguous with the upper two traces.

Characteristics of levcromakalim in the isolated uterus

Tissue bath studies Levcromakalim was markedly antagonized by the non-selective K-channel inhibitor TEA (10 mM). Functional antagonism, due to the spasmogenic action of TEA, may account for part of this antagonism. However, most of the antagonism seen must reflect selective interaction between TEA and levcromakalim as the antagonism was much greater than that against salbutamol or relaxin. Levcromakalim was also markedly antagonized by the purported K_{ATP}-selective K-channel inhibitor, glibenclamide (10 μM). Levcromakalim inhibited the uterine spasm evoked by low concentrations of KCl but not that evoked by high concentrations of KCl. Therefore, the observations from tissue bath studies are consistent with findings for this and other openers of K_{ATP}-channels in various smooth muscles (Edwards & Weston, 1994) including rat (Piper *et al.*, 1990) and human uterus (Cheuk *et al.*, 1993).

$^{42}K^+$ efflux studies In smooth muscles other than uterus, cromakalim is able to induce pronounced efflux of $^{42}K^+$ or its marker $^{86}Rb^+$ (Hamilton *et al.*, 1986; Quast & Baumlin, 1988; Edwards & Weston, 1994). Such data are somewhat lacking in uterine muscle. In longitudinal myometrium from the pregnant rat, cromakalim, at a concentration which inhibited myometrial spasm, did not alter the efflux of $^{42}K^+$ or $^{86}Rb^+$ (Hollingsworth *et al.*, 1987; 1989). Similarly, in longitudinal myometrium from the non-pregnant rat, cromakalim (10 μM) in normal PSS, promoted only a small and non-sustained increase in the efflux of $^{42}K^+$ and $^{86}Rb^+$ (Piper & Hollingsworth, 1995). However, a large $^{42}K^+$ efflux was induced by cromakalim (10 μM) in the presence of diltiazem (1 μM) and KCl (20 mM; Piper & Hollingsworth, 1995). This efflux was maintained for the duration of drug exposure and was partially inhibited by glibenclamide. These data suggest that the effect of cromakalim is dependent upon the K^+ gradient and/or the potential across the membrane (Piper & Hollingsworth, 1995).

Electrophysiological studies A characteristic property of levcromakalim and cromakalim in most smooth muscles is to produce marked hyperpolarization (Edwards & Weston, 1994). However, cromakalim (10 μM) induced a hyperpolarization of only 5 mV in uterus from the pregnant rat (Hollingsworth *et al.*, 1987). In the current study, levcromakalim (2 μM and 10 μM) failed to induce any hyperpolarization of uterine tissue which is in line with the small stimulation of efflux with cromakalim seen in normal PSS (Piper & Hollingsworth, 1995). One explanation could be that the resting membrane potential is close to the potassium equilibrium potential (K_{eq}) and hence K-channel opening would not result in significant hyperpolarization. However, the mean resting membrane potential recorded here (−63.3 mV), very close to the value determined by Kuriyama & Suzuki (1976; −65 mV), is much less negative than the K_{eq} calculated by Casteels & Kuriyama (1965; −77 to −86 mV). All values are from uteri from oestrogen-treated non-pregnant rats. Quast (1993) has drawn attention to several anomalies in the proposal that opening of K-channels in the plasma membrane is the sole mechanism to explain the relaxant properties of K_{ATP}-channel openers. It may be that levcromakalim and related compounds have a single biochemical mechanism which manifests itself as K_{ATP}-channel opening and hence hyperpolarization in most, but not all, smooth muscles (Edwards *et al.*, 1993). In a minority of tissues, including the uterus, these other mechanisms must predominate.

Characteristics of salbutamol in the isolated uterus

Tissue bath studies The K-channel inhibitor TEA (10 mM) antagonized salbutamol to a small extent, the degree of antagonism was much less than against levcromakalim and possibly reflects merely functional antagonism seen between a spasmogen and a relaxant. Salbutamol inhibited the spasm evoked by both high and low concentrations of KCl, a profile different from that of levcromakalim. This latter property of salbutamol could reflect its activation of a cyclic AMP-dependent pathway (Diamond, 1990).

$^{42}K^+$ efflux and electrophysiological studies Salbutamol inhibited the efflux of $^{42}K^+$ and $^{86}Rb^+$ from the myometrium of the non-pregnant rat (Piper, 1992) and in the current study did not induce hyperpolarization of uterine tissue. These data might be taken to indicate that salbutamol does not open K-channels. Other agonists at β-adrenoceptors have been shown to induce some hyperpolarization of the membrane of uterine muscle (Diamond & Marshall, 1969; Kroeger & Marshall, 1973), which was attributed to an increase in K^+ conductance (Marshall, 1977; Anwer *et al.*, 1992). These K-channel opening actions have been suggested to have a supportive rather than central role in the action of β-adrenoceptor agonists (Diamond & Marshall, 1969; Cook *et al.*, 1993). Although the electrophysiological profile of salbutamol in rat uterus was not different from that of levcromakalim, the characteristics of salbutamol were markedly different from those of levcromakalim in the tissue bath experiments.

Characteristics of relaxin in the isolated uterus

Tissue bath studies Relaxin was a potent inhibitor of the spasm evoked by oxytocin (0.2 nM) in the uterus from the non-pregnant rat. Relaxin was slightly antagonized by TEA, an interaction probably a consequence of functional antagonism, and was not antagonized by glibenclamide. Relaxin also reduced the size of the spasm evoked by all concentrations of KCl. In this respect relaxin was more like salbutamol than levcromakalim. Such a profile of action against the spasm evoked by low and high concentrations of KCl is not characteristic of an agent which acts via the opening of K-channels. The profile of action of relaxin in the presence of these inhibitors was similar to that of salbutamol and not at all like that of levcromakalim. These data do not support the involvement of K-channel opening in the mechanism of action of relaxin.

The lack of antagonism of relaxin by glibenclamide in the current *in vitro* study contrasts with the large (19 fold) and selective antagonism of relaxin, as a uterine relaxant, by glibenclamide *in vivo* (Downing & Hollingsworth, 1991). This contrast was a most surprising finding as the *in vivo* results were the stimulus for this study. There are several differences between the hormonal and spasmogenic conditions in the *in vivo* and *in vitro* experiments, but these differences do not appear to account for the contrast (Hughes & Hollingsworth, 1994). It may be that either glibenclamide or relaxin have different mechanisms of action *in vivo* compared to *in vitro* in the rat uterus.

$^{42}K^+$ efflux and electrophysiological studies In normal PSS relaxin did not increase the efflux rate of $^{42}K^+$. Our observation that, in the presence of diltiazem and KCl, conditions under which cromakalim induced a modest, sustained efflux of $^{42}K^+$ (Piper & Hollingsworth, 1995), relaxin stimulated only a very small and non-sustained $^{42}K^+$ efflux further indicates that relaxin does not have a K-channel opening action. Relaxin did not hyperpolarize the tissue; in this respect relaxin was like both levcromakalim and salbutamol. The relaxin-induced decrease in spiking activity but lack of hyperpolarization is in agreement with previous studies (Chamley & Parkington, 1984; Osa *et al.*, 1991). Thus, comparison of the actions of relaxin on $^{42}K^+$ efflux *in normal* PSS and on membrane potential with those of salbutamol and levcromakalim neither supports nor opposes the hypothesis of the involvement of K-channel opening in the mechanism of action of relaxin. Anwer *et al.* (1993) have shown that recombinant human relaxin stimulates the opening of a high-conductance Ca^{2+}-activated K-

channel in an immortalized human myometrial cell line. Such findings are difficult to reconcile with mechanical studies as human relaxin is inactive as a relaxant of isolated myometrial strips from human subjects (MacLennan, 1994).

In summary, relaxin, like salbutamol but unlike levcromakalim, did not selectively inhibit the spasm evoked by low concentrations of KCl and was not markedly antagonized by TEA or by glibenclamide. Under conditions where it was possible to demonstrate a cromakalim-induced increase in the $^{42}K^+$-efflux rate, relaxin had no effect. Thus, the actions of relaxin were not characteristic of a compound which acts via the opening of K-channels. Relaxin may have a K-channel opening action which is localized at pacemaker regions and induces changes in $^{42}K^+$ flux which are too small or too localized to be detected by the efflux and electrophysiological techniques applied here. If relaxin does exhibit such an action, it does not involve glibenclamide-sensitive K-channels as does levcromakalim. Although relaxin does not appear to act via the opening of K-channels, its primary site of action appears to be located in the plasma membrane (Hughes & Hollingsworth, 1995).

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