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Cellular localization of the inhibitory action of relaxin against uterine spasm

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1 The aim of this study was to determine whether the site of action of relaxin as a relaxant of rat myometrium is at the cell membrane or at an intracellular site. Therefore, the potency of relaxin was determined against spasms reliant predominantly upon either extracellular Ca^{2+} or intracellular Ca^{2+}. Uterine spasms dependent upon extracellular Ca^{2+} were elicited by (i) oxytocin (0.2 nM) (ii) Bay K 8644 (1 μM) in 10 mM K^+-rich PSS and (iii) KCl (80 mM). Uterine spasm dependent upon intracellular Ca^{2+} was elicited by oxytocin (20 nM) in the presence of nifedipine (500 nM). The effects of relaxin against these spasmogens were compared with those of levcromakalim, nifedipine and salbutamol.

2 Relaxin (0.2-6.3 nM), levcromakalim (25-800 nM), salbutamol (1-63 nM) and nifedipine (1-250 nM) caused concentration-dependent inhibition of the spasm evoked by oxytocin (0.2 nM) and relaxin was the most potent relaxant.

3 Relaxin and nifedipine were slightly less potent against the spasm induced by Bay K 8644 (1 μM) than against spasm induced by oxytocin (0.2 nM) (15 fold and 13 fold respectively). Levcromakalim and salbutamol were equipotent against the spasm evoked by Bay K 8644 (1 μM) and that evoked by oxytocin (0.2 nM).

4 Relaxin induced only $47 \pm 7\%$ inhibition of the KCl (80 mM)-evoked spasm at a concentration of 0.8 μM. Levcromakalim was much less potent (427 fold) against the spasm evoked by KCl (80 mM) than against the spasm evoked by oxytocin (0.2 nM). The potency of salbutamol against the spasm evoked by KCl (80 mM) was modestly reduced (14 fold) compared to that against the spasm evoked by oxytocin (0.2 nM). The potency of nifedipine against the KCl (80 mM)-evoked spasm was not different from that against the oxytocin (0.2 nM)-evoked spasm.

5 The potencies of relaxin and levcromakalim against the spasm evoked by oxytocin (20 nM) + nifedipine (500 nM) were greatly reduced (74 fold and 234 fold respectively) compared to their potencies against the spasm evoked by oxytocin (0.2 nM). The potency of salbutamol against these two spasmogens was not different.

6 Relaxin was much less potent against the spasm dependent upon intracellular Ca^{2+} (that induced by oxytocin (20 nM) + nifedipine (500 nM)) than against the spasms dependent upon extracellular Ca^{2+}, those induced by oxytocin (0.2 nM) and Bay K 8644 (1 μM). In this regard, relaxin resembled levcromakalim and nifedipine rather than salbutamol. Therefore, the major site of action of relaxin appears to be located at the plasma membrane rather than at an intracellular level. The observation that relaxin was less effective against the KCl (80 mM)-induced spasm than against the oxytocin (0.2 nM)-evoked spasm may indicate that relaxin has a minor action involving K^+-channel opening.

7 High concentrations of relaxin (up to 1 μM) induced significant inhibition of the spasm dependent upon intracellular Ca^{2+}. Thus at high concentrations relaxin also appears to have an additional intracellular action.

Keywords: Relaxin; uterus; intracellular Ca^{2+}; extracellular Ca^{2+}; levcromakalim; salbutamol; nifedipine; oxytocin; Bay K 8644; potassium chloride

Introduction

Relaxin is one of the most potent known relaxants of uterine smooth muscle (Downing & Hollingsworth, 1993). It is a polypeptide hormone mainly derived from the corpus luteum and has a role in maintaining quiescence of the uterus during pregnancy. The two mechanisms which have been proposed to explain the inhibitory action of relaxin in the uterus are located at different sites in the cell. There is evidence that relaxin may act via a cell surface receptor to activate the adenylyl cyclase pathway and stimulate an adenosine 3':5'-cyclic monophosphate (cyclic AMP)-dependent protein kinase which in turn modulates intracellular enzymes and proteins (Sanborn & Anwer, 1990; Downing & Hollingsworth, 1993). Relaxin has been shown to increase the uterine concentration of cyclic AMP (Cheah & Sherwood, 1980; Judson *et al.*, 1980; Sanborn *et al.*, 1980; Hsu *et al.*, 1985; Osa *et al.*, 1991) and to increase the activity of cyclic AMP-dependent protein kinase A (Kemp & Niall, 1981; Hughes & Hollingsworth, 1994). However, the changes in uterine cyclic AMP concentrations produced by relaxin are small and tend to follow rather than precede the mechanical inhibition (Downing *et al.*, 1992).

Alternatively, relaxin has been proposed to act at the level of the plasma membrane and open potassium (K^+)-channels (Downing & Hollingsworth, 1991). *In vivo*, relaxin, as a uterine relaxant, was selectively antagonized by glibenclamide, the purported blocker of ATP-sensitive K^+-channels (Cook & Quast, 1990). The relative importance of these two mechanisms in the action of relaxin is uncertain and it may be that a different mechanism altogether is involved. Clearly to elucidate the mechanism by which relaxin inhibits uterine mechanical activity it is necessary first to determine whether relaxin modulates events localized in the plasma membrane or whether it acts via a more intracellular site to block tension development.

Relaxants that modulate events in the plasma membrane and prevent calcium (Ca^{2+}) influx should inhibit spasm(s) that

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are reliant upon extracellular Ca^{2+} but should be relatively ineffective against spasm(s) that result from the release of intracellular Ca^{2+}. On the other hand, relaxants that modulate events within the cell, further down the biochemical cascade leading to tension development, should inhibit both spasms that are dependent upon extracellular Ca^{2+} and those that are dependent upon intracellular Ca^{2+}. Therefore, the potency of relaxin was determined against spasms reliant upon extracellular Ca^{2+} and intracellular Ca^{2+}. Uterine spasms dependent upon extracellular Ca^{2+} were elicited by (i) low concentrations of oxytocin (Ashoori *et al.*, 1985; Edwards *et al.*, 1986; Granger *et al.*, 1986), (ii) the Ca^{2+}-channel agonist, Bay K 8644 (Poli *et al.*, 1989; Calixto & Rae, 1991) and (iii) iso-osmolar 80 mM KCl (Granger *et al.*, 1986). These spasms were abolished in Ca^{2+}-free media (oxytocin: Edwards *et al.*, 1986; Bay K 8644; Kyozuka *et al.*, 1987; KCl: Granger *et al.*, 1986). Uterine spasm dependent upon intracellular Ca^{2+} was elicited by high concentrations of oxytocin (Edwards *et al.*, 1986; Marc *et al.*, 1986) in the presence of the Ca^{2+} channel blocker nifedipine (Triggle & Rampe, 1989). Significant spasm to oxytocin (20 nM) remained in Ca^{2+}-free medium (Ashoori *et al.*, 1985). The effects of relaxin against these spasmogens were compared with those of levcromakalim, a K^+-channel opener (Weston & Edwards, 1992), and nifedipine, a Ca^{2+}-channel blocker (Triggle & Rampe, 1989), both of which influence events in the plasma membrane. The effects of relaxin were also compared with those of salbutamol, a β-adrenoceptor agonist, which acts via a G-protein-coupled cell surface receptor to stimulate the adenylyl cyclase pathway (Diamond, 1990). Preliminary results have been presented to the British Pharmacological Society (Hughes & Hollingsworth, 1993).

Methods

Tissue preparation and tension recording

Female, non-pregnant Sprague-Dawley rats (200–300 g) were purchased from Charles River Ltd. Rats were pretreated 18–24 h prior to experimentation with 17β-oestradiol benzoate (100 μg kg^{-1}, s.c.). Rats were killed and uterine horns removed and placed in a physiological salt solution (PSS). Each horn was cut into two segments of approximately 1 cm length which were mounted in 10 ml tissue baths for the isometric recording of tension changes, primarily from the longitudinal muscle layer. Tissues were allowed to equilibrate under 1 g tension in PSS maintained at 37°C and gassed with 95% O_2/5% CO_2 for 30–60 min prior to experimentation. Mechanical responses were measured as the integral of the tension versus time curve by the method of Granger *et al.* (1985) in units of g s, usually over 5 min periods.

Protocol

Following equilibration, the tissues were exposed to one of the following spasmogens: (a) oxytocin (0.2 nM); (b) Bay K 8644 (1 μM) in 10 mM K^+-rich PSS; (c) iso-osmolar KCl (80 mM); (d) oxytocin (20 nM), following preincubation with nifedipine (500 nM) for 40 min and in its continued presence. The uteri were exposed to the spasmogen for 10 (c,d) or 15 (a,b) min before relaxant (levcromakalim, salbutamol, nifedipine or relaxin) was added to the tissue bath in the continued presence of the spasmogen. A single concentration-effect curve to a relaxant was constructed for 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 order to determine whether the spasms were well-maintained for the duration of the construction of the curve, the tension induced by each spasmogen was monitored in separate tissues from the same animals to which no relaxant was added. The integral of the spasm evoked by oxytocin (0.2 nM) was well-maintained during the time course of the experiment. The integral of the spasm induced by Bay K 8644 (1 μM), by KCl (80 mM) and by oxytocin (20 nM) declined slightly with time (see Results).

In each segment of tissue, for each concentration of relaxant applied, the integral of the tension recorded from 5 to 10 min after relaxant addition was expressed as a percentage of the integral of the tension recorded in the 5 min period prior to the administration of relaxant, to determine the % spasm remaining. For groups (b), (c) and (d) the relaxant-induced effects were adjusted to account for the decline of the spasm in time-matched controls. The mean % spasm remaining was calculated at each time point in the time-matched controls. For each relaxant-exposed tissue the % spasm remaining at each relaxant concentration was expressed as a percentage of the mean % spasm remaining at the corresponding time point in the time-matched controls. pD_2 values were calculated for each segment of tissue from the regression line fitted to the plot of probit response versus $\log_{10}$ molar relaxant concentration (Tallarida & Murray, 1981; Foster & Hollingsworth, 1985). The pD_2 values were averaged for each experimental group to give the mean $pD_2 \pm$ s.e.mean. The differences in potencies of the relaxants against spasmogens (b)-(d) above compared to their potencies against the spasm evoked by oxytocin (0.2 nM, a) 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 graphical purposes the mean $\pm$ s.e.mean % spasm remaining was calculated for each relaxant concentration to give the mean $\log_{10}$ concentration-inhibition curve. Due to the comparative nature of this work the effects of the relaxants against the spasm taken as standard, that induced by oxytocin (0.2 nM), are presented on each of the three graphs. Statistical comparison of mean pD_2 values was with a Student's two-tailed, unpaired *t* test with significance identified at $P < 0.05$.

Drugs and solutions

The following drugs were used: levcromakalim (SmithKline Beecham); salbutamol sulphate (Glaxo); nifedipine, Bay K 8644 (methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate, Bayer); oxytocin acetate and 17β-oestradiol benzoate (Sigma); KCl (BDH Chemicals). 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 porcine relaxin preparation was found to be equipotent with highly purified porcine relaxin. Stock solutions were prepared in the following solvents: levcromakalim, 70% v/v ethanol; salbutamol, 0.1 M HCl; relaxin, saline; nifedipine, Bay K 8644, oxytocin acetate, KCl, twice-distilled water; oestradiol benzoate, arachis oil. Serial dilutions of the stock dilutions were made in saline on the day of use. Experiments with nifedipine were performed under sodium lamp illumination due to the light-sensitive nature of this compound.

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 composition of the K^+-rich PSS was modified from that above in order to maintain osmolarity (mM), 10 mM K^+-rich PSS: Na^+ 138.9, K^+ 10.0; 80 mM K^+-rich PSS: Na^+ 68.9, K^+ 80.0.

Results

Relaxant inhibition of spasm evoked by oxytocin (0.2 nM)

Oxytocin (0.2 nM) induced a regular, phasic spasm (Figure 1a) that was well-maintained for 2 h. The integral of the spasm in the 5 min period before relaxant addition was 313 ± 23 ($n = 28$) g s. Relaxin (0.2–6.3 nM), levcromakalim (25–800 nM), sal-

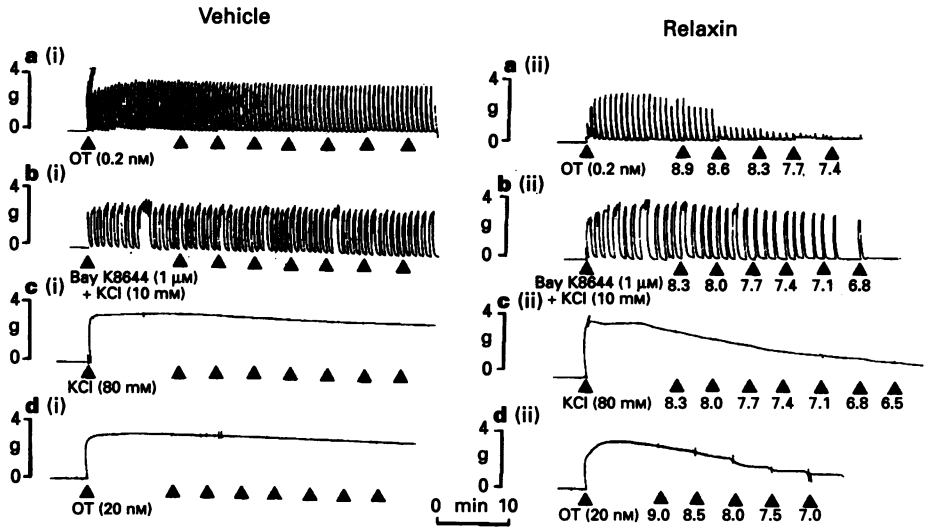

Figure 1 Illustration of spasms elicited by oxytocin (OT, 0.2 nM; a), Bay K 8644 (1 μM) in the presence of KCl (10 mM; b), KCl (80 mM; c) and oxytocin (20 nM) after preincubation with nifedipine (500 nM) for 40 min (d). Shown are the effects of cumulative addition of relaxin (ii) and solvent (saline, i).

butamol (1–63 nM) and nifedipine (1–250 nM) caused concentration-dependent inhibition of the spasm evoked by oxytocin (0.2 nM), with maximal effects close to 100% inhibition (Figure 1–4). The pD_2 value for each relaxant against this spasmogen is shown in Table 1. Relaxin was the most potent relaxant against the spasm induced by oxytocin (0.2 nM).

Relaxant inhibition of spasm evoked by Bay K 8644 (1 μM) in K^+-rich PSS

Bay K 8644 (1 μM) induced a regular, phasic spasm in 10 mM K^+-rich PSS (Figure 1b), the integral of this spasm declined by 17% over 1 h 40 min. The integral of spasm in the 5 min period before relaxant addition was 764±44 (n=31) g s. Relaxin (0.8 nM–0.8 μM) inhibited this spasm in a concentration-dependent manner but with a maximum effect of 85.0±5.7% inhibition at 0.8 μM (Figures 1b(ii), 2b). Levcromakalim (0.05–6.3 μM), salbutamol (0.5–250 nM) and nifedipine (0.01–1.3 μM) all caused concentration-dependent inhibition of the spasm evoked by Bay K 8644 (1 μM) in K^+-rich PSS (Figure 2).

The potencies of relaxin and nifedipine against the spasm induced by Bay K 8644 (1 μM) were reduced by a similar degree (15 fold and 13 fold respectively; $P<0.01$) compared to their potencies against the spasm induced by oxytocin (0.2 nM) (Table 1). Levcromakalim and salbutamol were equipotent against the spasm evoked by Bay K 8644 (1 μM) and that evoked by oxytocin (0.2 nM) (Table 1).

Relaxant inhibition of spasm evoked by KCl (80 mM)

Iso-osmolar K^+ (80 mM)-enriched PSS induced a tonic spasm (Figure 1c), the integral of this spasm declined by 23% over 1 h 40 min. The integral of spasm in the 5 min period before relaxant addition was 1507±73 (n=36) g s. Relaxin induced only 47±7% inhibition of the KCl (80 mM)-evoked spasm at a concentration of 0.8 μM (Figure 3b). Levcromakalim (2–500 μM), salbutamol (0.001–32 μM) and nifedipine (2–130 nM) caused concentration-dependent inhibition of the spasm evoked by iso-osmolar KCl (80 mM) (Figure 3). Most of the inhibition by levcromakalim at concentrations above 100 μM is likely to be due to the effect of the ethanol vehicle rather than of the drug itself (see Figure 3a).

The potency of relaxin against the KCl (80 mM)-evoked spasm was greatly reduced compared to that against the oxytocin (0.2 nM)-evoked spasm (more than 100 fold; Table 1).

Table 1 The potencies (pD_2) and $\log_{10}$ concentration ratios of 4 relaxants against different spasmogens in isolated uterus of the non-pregnant rat

		Spasmogens			
		Oxytocin (0.2 nM)	*Oxytocin* (20 nM) + *nifedipine* (500 nM)	*Bay K 8644* (1 μM) + 10 mM K^+ *rich PSS*	*KCl* (80 mM)
Relaxin	pD_2	9.15±0.16	7.24±0.25**	7.98±0.23*	Only 47±7% inhibition at 0.8 μM
	$\log_{10}$ CR	–	1.87±0.30	1.17±0.28	–
	mean CR	–	74	14.8	>100
Levcromakalim	pD_2	6.58±0.32	4.21±0.17**	7.06±0.21	3.95±0.09**
	$\log_{10}$ CR	–	2.37±0.36	–0.48±0.38	2.63±0.33
	mean CR	–	234	–3.0	427
Salbutamol	pD_2	8.14±0.12	7.98±0.10	8.48±0.16	6.98±0.14**
	$\log_{10}$ CR	–	0.16±0.16	–0.34±0.20	1.16±0.18
	mean CR	–	1.4	–2.2	14
Nifedipine	pD_2	7.86±0.12	–	6.74±0.13**	8.01±0.13
	$\log_{10}$ CR	–	–	1.12±0.18	–0.15±0.18
	mean CR	–	–	13.2	–1.4

pD_2 and $\log_{10}$ CR values are means±s.e.mean, n=6–8. $\log_{10}$ CR±s.e.mean values represent potency vs oxytocin (20 nM), Bay K 8644 (1 μM) or KCl (80 mM) compared to potency vs oxytocin (0.2 nM). Mean CR=anti-log of $\log_{10}$ CR. *$P<0.01$, **$P<0.001$ indicate a significant difference from the pD_2 value attained against the oxytocin (0.2 nM)-induced spasm.

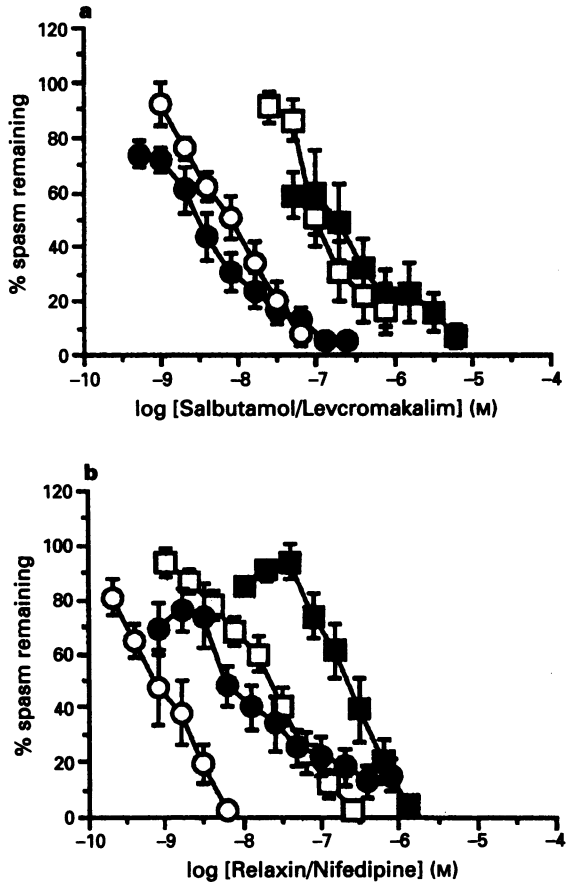

Figure 2 The effects of (a) salbutamol (○, ●) and levcromakalim (□, ■) and (b) relaxin (○, ●) and nifedipine (□, ■) on the spasms evoked by oxytocin (0.2 nM) (open symbols) and Bay K 8644 (1 μM) in K^+-rich (10 mM) PSS (closed symbols) in isolated uterus from the 17β-oestradiol benzoate-pretreated, non-pregnant rat. Ordinate scales: % spasm remaining represents the integral of the tension recorded from 5 to 10 min after the addition of relaxant expressed as a percentage of the integral of the tension recorded for 5 min prior to any relaxant addition. Abscissa scales: $\log_{10}$ molar concentration of relaxant. Points represent the means ± s.e.mean of $n = 6-8$ tissues.

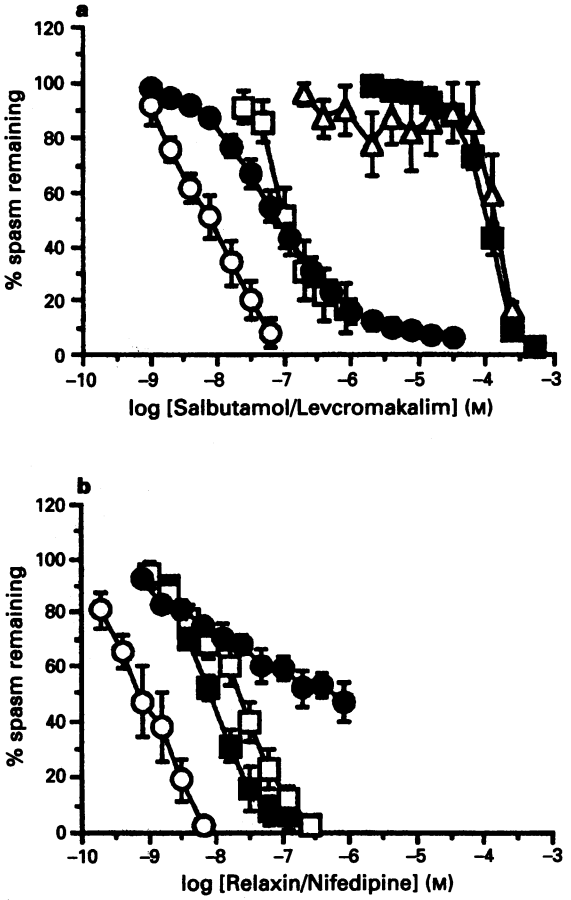

Figure 3 The effects of (a) salbutamol (○, ●) and levcromakalim (□, ■) and (b) relaxin (○, ●) nifedipine (□, ■) on the spasms evoked by oxytocin (0.2 nM) (open symbols) and KCl (80 mM) (closed symbols) in the isolated uterus from the 17β-oestradiol benzoate-pretreated, non-pregnant rat. In a separate set of tissues ethanol, the vehicle for levcromakalim, was applied at concentrations equivalent to those applied when constructing a concentration-inhibition curve for levcromakalim (a, △). Ordinate scales: % spasm remaining represents the integral of the tension recorded from 5 to 10 min after the addition of relaxant expressed as a percentage of the integral of the tension recorded for 5 min prior to any relaxant addition. Abscissa scales: $\log_{10}$ molar concentration of relaxant. Points represent the means ± s.e.mean of $n = 6-8$ tissues.

Levcromakalim was much less potent against the spasm evoked by KCl (80 mM) than against the spasm evoked by oxytocin (0.2 nM) (427 fold; $P < 0.001$; Table 1). The potency of salbutamol against the spasm evoked by KCl (80 mM) was modestly reduced compared to that against the spasm evoked by oxytocin (0.2 nM) (14 fold; $P < 0.001$; Table 1). The potency of nifedipine against the KCl (80 mM)-evoked spasm was not different from that against the oxytocin (0.2 nM)-evoked spasm ($P > 0.05$; Table 1).

Relaxant inhibition of spasm evoked by oxytocin (20 nM) + nifedipine (500 nM)

Oxytocin (20 nM), in the presence of nifedipine (500 nM), induced a tonic spasm (Figure 1d), the integral of this spasm declined by 49% over 1 h 40 min. The integral of spasm in the 5 min period before relaxant addition was 870 ± 48 ($n = 24$) g s. Relaxin (0.16 nM – 1.6 μM) inhibited this spasm in a concentration-dependent manner but with a maximum effect of only 80.0 ± 4.7% inhibition at 1.6 μM (Figure 1d(ii), 4b). Levcromakalim (0.002 – 1 mM) and salbutamol (1 – 500 nM) caused concentration-dependent inhibition of the spasm evoked by oxytocin (20 nM) in the presence of nifedipine (500 nM) (Figure 4a). High concentrations of ethanol vehicle, added when applying very high concentrations of levcromakalim, itself had a pronounced inhibitory effect (Figure 4a).

The potencies of relaxin and levcromakalim against the spasm evoked by oxytocin (20 nM) + nifedipine (500 nM) were greatly reduced (74 fold and 234 fold respectively; $P < 0.001$) compared to their potencies against the spasm evoked by oxytocin (0.2 nM) (Table 1). The potency of salbutamol against these two spasmogens did not differ (Table 1).

Discussion

This study examined the relative ability of relaxin to inhibit uterine spasm that was predominantly dependent upon either extracellular or intracellular Ca^{2+} in order to determine whether the primary site of action of relaxin is localized within the cell or at the level of the plasma membrane. Relaxin was able to inhibit spasms dependent upon both extracellular Ca^{2+} and intracellular Ca^{2+} but it was more potent against the former than the latter. Thus, the action of relaxin appears to be located primarily at the level of the plasma membrane.

Nature of the spasms

The phasic spasm elicited by low concentrations of oxytocin is probably dependent mainly upon extracellular Ca^{2+}. Low concentrations of oxytocin have been shown to increase the influx of Ca^{2+} across the plasma membrane (Ashoori *et al.*,

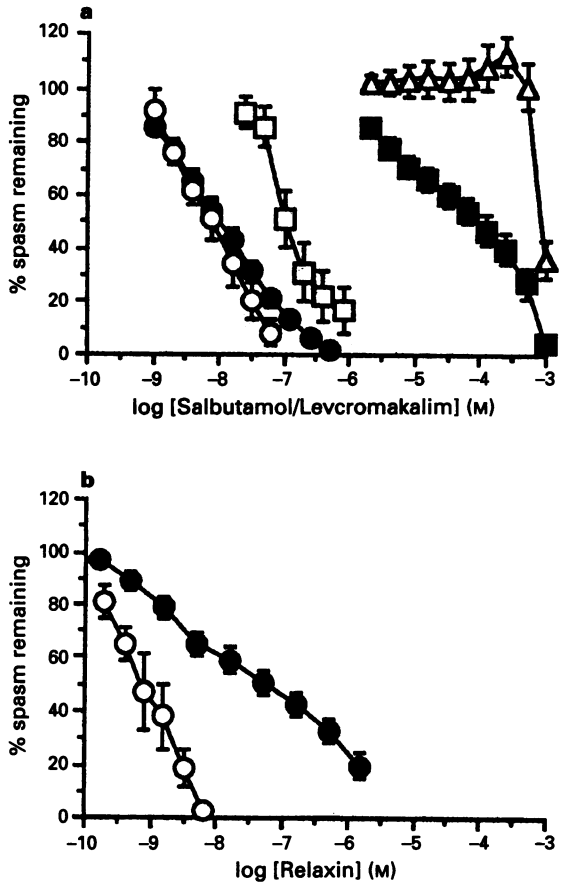

Figure 4 The effects of (a) salbutamol (○, ●) and levcromakalim (□, ■) and (b) relaxin (○, ●) on the spasms evoked by oxytocin (0.2 nM) (open symbols) and oxytocin (20 nM) + nifedipine (500 nM) (closed symbols) in isolated uterus from the 17β-oestradiol benzoate-pretreated, non-pregnant rat. In a separate set of tissues ethanol, the vehicle for levcromakalim, was applied at concentrations equivalent to those applied when constructing a concentration-inhibition curve for levcromakalim (a, △). Ordinate scales: % spasm remaining represents the integral of the tension recorded from 5 to 10 min after the addition of relaxant expressed as a percentage of the integral of the tension recorded for 5 min prior to any relaxant addition. Abscissa scales: $\log_{10}$ molar concentration of relaxant. Points represent the means ± s.e.mean of $n = 6-8$ tissues.

1985; Edwards *et al.*, 1986; Granger *et al.*, 1986) and to enhance the inward Ca^{2+} current (Mironneau, 1976). Both the spasm and Ca^{2+} influx induced by low concentrations of oxytocin are abolished by L-type Ca^{2+}-channel blockers or by removing extracellular Ca^{2+} (Edwards *et al.*, 1986).

Bay K 8644 is a dihydropyridine Ca^{2+}-channel agonist (Triggle & Rampe, 1989) which induces uterine spasm that is abolished by Ca^{2+}-channel blockers and by the absence of extracellular Ca^{2+} indicating that this spasm is dependent upon extracellular Ca^{2+} (Conte-Camerino *et al.*, 1987; Kyozuka *et al.*, 1987). Application of Bay K 8644, in normal PSS, will not induce a well maintained spasm in uterine tissue. Most studies in rat uterus have shown that Bay K 8644 requires raised K^+ concentrations to induce spasm (Conte-Camerino *et al.*, 1987; Kyozuka *et al.*, 1987) and $^{45}Ca^{2+}$ uptake (Ichida *et al.*, 1991). In this study it was shown that only a small rise in the K^+ concentration (total K^+ 10 mM) was required for Bay K 8644 to induce a well maintained phasic spasm. Under these conditions of partial depolorization it is thought that the inactive Ca^{2+}-channels are converted to the open, active state (Triggle & Rampe, 1989).

Spasm elicited by KCl (80 mM) is probably dependent mainly upon extracellular Ca^{2+}. The tonic contraction induced by this spasmogen has been shown to be associated with the influx of Ca^{2+} across the plasma membrane (Granger *et al.*, 1986). L-type Ca^{2+}-channel blockers and zero extracellular Ca^{2+} conditions abolish both the spasm evoked by KCl (80 mM) and the associated Ca^{2+} influx (Granger *et al.*, 1986).

Spasm dependent upon intracellular Ca^{2+} was elicited by oxytocin (20 nM) in the presence of the Ca^{2+}-channel blocker, nifedipine (500 nM). The spasm induced by high concentrations of oxytocin is not abolished by lowering the extracellular Ca^{2+} concentration indicating that at these high concentrations oxytocin probably releases Ca^{2+} from the intracellular stores (Ashoori *et al.*, 1985; Edwards *et al.*, 1986). Inositol trisphosphate has been shown to be involved in this action (Marc *et al.*, 1986; 1988). Nifedipine was added to prevent influx of Ca^{2+} from the extracellular fluid via L-type Ca^{2+}-channels, and so ensure that the spasm was dependent mainly upon intracellular Ca^{2+}.

Effects of levcromakalim

Levcromakalim was used as a standard drug whose site of action is thought to be within or close to the plasma membrane. It is proposed that levcromakalim opens K^+-channels and hyperpolarizes the plasma membrane, an action thought to deactivate voltage-dependent Ca^{2+}-channels and inhibit spasm (Weston & Edwards, 1992). As expected, levcromakalim was of similar high potency against the two spasmogens with actions dependent mainly upon extracellular Ca^{2+}, oxytocin (0.2 nM) and Bay K 8644 (1 μM). As anticipated, levcromakalim was of much lower potency against the spasm evoked by KCl (80 mM) (Piper *et al.*, 1990). This observation, characteristic of this class of drug, relates to the fact that under these conditions the potassium equilibrium potential is more positive than the potential at which the Ca^{2+}-channels are opened, hence the hyperpolarization induced by the K^+-channel openers is insufficient to deactivate the Ca^{2+}-channels and inhibit the associated spasm.

Levcromakalim was much less potent against the spasm dependent mainly upon intracellular Ca^{2+} (that evoked by oxytocin (20 nM) in the presence of nifedipine (500 mM)) than against the spasm evoked by oxytocin (0.2 nM). This observation is compatible with the proposed mechanism of action of levcromakalim and supports the idea that inhibition of the former spasm is diagnostic of an agent acting via intracellular mechanisms.

At high concentrations (> 60 μM), levcromakalim was observed to inhibit the KCl (80 mM)-evoked spasm, as seen previously with cromakalim (Piper *et al.*, 1990). Also, some inhibition of the oxytocin (20 nM)-evoked spasm was produced by levcromakalim at concentrations > 10 μM. At these concentrations these effects are likely to be attributable in part to the ethanol vehicle. However, in rat uterus very high concentrations of cromakalim appear to have an action which may not involve K^+-channel opening (Piper *et al.*, 1990). Indeed, in other tissues it has been suggested that levcromakalim-induced membrane hyperpolarization is linked to the modulation of intracellular Ca^{2+} stores and that levcromakalim can modulate intracellular events via mechanisms not linked to the opening of K^+-channels in the plasma membrane (Greenwood & Weston, 1993; Quast, 1993).

Effects of nifedipine

Nifedipine was used as a standard drug whose mechanism of action involves inhibition of Ca^{2+} influx from the extracellar medium. The observations that nifedipine inhibited the spasms induced by oxytocin (0.2 nM) and by KCl are compatible with this mechanism. The reduced potency of nifedipine against the Bay K 8644-induced spasm relative to its potency against the oxytocin (0.2 nM)-induced spasm was also expected on the basis of the competitive interaction seen between Bay K 8644 and nifedipine (Asano *et al.*, 1987; Conte-Camerino *et al.*, 1987; Kyozuka *et al.*, 1987). It was clearly not feasible to assess the potency of nifedipine against the model of spasm dependent on intracellular Ca^{2+}, oxytocin (20 nM) + nifedipine (500 nM).

Effects of salbutamol

Salbutamol was used as a standard drug whose mechanism of action involves a cell surface receptor coupled via a G-protein to adenylyl cyclase, and results in an increase in the concentration of cyclic AMP and activation of protein kinase A (Diamond, 1990). Since protein kinase A has wide ranging effects, salbutamol was expected to inhibit various spasmogens which employ different mechanisms to induce spasm. Salbutamol was indeed equipotent against the spasms induced by oxytocin (0.2 nM), by Bay K 8644 (1 μM) and by oxytocin (20 nM) + nifedipine (500 nM). Clearly salbutamol was equipotent against spasms dependent upon extracellular and intracellular Ca^{2+}.

Salbutamol was slightly less potent against the spasm evoked by KCl (80 mM). Salbutamol was able to cause complete inhibition of this spasm which indicates that the main action of salbutamol is not attributable to the opening of K^+-channels. However, the reduced potency of salbutamol against this spasm may indicate a minor K^+-channel opening action as has been suggested for β-adrenoceptor agonists in uterus (Diamond & Marshall, 1969; Marshall, 1977; Anwer *et al.*, 1992) and in trachealis (Cook *et al.*, 1993). Such actions have been suggested to have a supportive rather than a central role. Alternatively, such a decrease in potency may be a consequence of functional antagonism due to the degree of tone induced by the KCl (Cook *et al.*, 1995).

Effects of relaxin

Relaxin was the most potent of the four relaxants against the spasm induced by oxytocin (0.2 nM). Relaxin was less potent against the spasm induced by oxytocin (20 nM) + nifedipine (500 nM), which was dependent upon intracellular Ca^{2+}, than against the spasms induced by oxytocin (0.2 nM) or Bay K 8644 (1 μM), which were dependent upon extracellular Ca^{2+}. In this regard, relaxin resembled levcromakalim more than salbutamol. Thus, the major action of relaxin appears to be located at the plasma membrane rather than at an intracellular site. Although relaxin inhibited both the spasms induced by oxytocin (0.2 nM) and by Bay K 8644 (1 μM), it was 15 fold less potent against the latter spasmogen than against the former. In this regard relaxin was very similar to nifedipine. Since the action of nifedipine is located at the plasma membrane this observation further supports the suggestion that relaxin modulates events localized in the plasma membrane and might even be taken to indicate that relaxin modulates L-type Ca^{2+}-channel activity.

Relaxin has been suggested to induce relaxation of uterus by a cell-surface receptor coupled via a pertussis toxin-sensitive G-protein to adenylyl cyclase (Sanborn & Anwer, 1990). However, relaxin-induced increases in myometrial cyclic AMP (Downing *et al.*, 1992) and protein kinase A (Hughes & Hollingsworth, 1994) are small. The current functional results do not support the contention that this mechanism is fundamental to the uterine relaxant action of low concentrations of relaxin.

With most of the evidence suggesting that relaxin modulates events localized in the plasma membrane it was surprising to find that relaxin could induce a maximum inhibition of only 47% of the spasm evoked by KCl (80 mM). Such an action might be indicative of a K^+-channel opening action of relaxin (Downing & Hollingsworth, 1991; Hughes *et al.*, 1992) or a consequence of functional antagonism due to the degree of tone induced by the KCl. The integral of spasm induced by KCl was greater than that produced by the other spasmogens.

Higher concentrations of relaxin (0.16 nM – 1.6 μM) did inhibit the spasm evoked by oxytocin (20 nM) + nifedipine (500 nM) but the slope of the concentration-effect curve was less than that against oxytocin (0.2 nM) and the maximum effect was 80% inhibition at 1.6 μM. This observation is compatible with a mechanism involving intracellular actions. It is possible that the data supporting adenylyl cyclase stimulation are relevant to these concentrations of relaxin.

In summary, the primary site of action of low concentrations of relaxin in rat uterus appears to be located at the plasma membrane. At high concentrations, relaxin appears to exert an additional action involving an intracellular mechanism.

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