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Tocolytic Effects of a Long-acting β_2-Adrenoceptor Agonist, Formoterol, in Rats

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Abstract

We have assessed the tocolytic activity of formoterol, a novel long-acting and potent β_2-adrenoceptor agonist, through its production of cyclic adenosine monophosphate, in comparison with ritodrine, a β_2-adrenoceptor agonist used clinically to counter premature delivery.

Formoterol and ritodrine inhibited the amplitude and frequency of rat uterine contraction, with IC50 values of $3{\cdot}8 \times 10^{-10}$ and $4{\cdot}7 \times 10^{-7}$ M, respectively. Intravenous administration of formoterol or ritodrine caused inhibition of uterine motility and increased heart rate in a dose-dependent manner. Inhibition of uterine motility by oral administration of formoterol (0·3 and 1 mg kg^{-1}) continued for at least 60 min, whereas that with ritodrine (100 mg kg^{-1}) persisted for 15 min with rapid recovery thereafter in pregnant rats. The β-adrenoceptor binding of [^{125}I]iodopindolol to the myometrium of pregnant rats was competitive with formoterol and ritodrine, with K_i values of 0·04 and 6·10 nM, respectively. Formoterol (10^{-6}–10^{-4} M) and ritodrine (10^{-6}–10^{-4} M) increased the level of cyclic adenosine monophosphate in lymphocytes in a dose-dependent manner.

The results suggested that formoterol caused relaxation of uterine motility through production of cyclic adenosine monophosphate. Thus, formoterol may be useful as a treatment to counter premature delivery.

Ritodrine and other β_2-adrenoceptor agonists are often used as uterine relaxants to prevent premature delivery. Stimulation of β-adrenoceptors in the myometrium results in an increase of cellular cyclic adenosine monophosphate (cAMP) through the activation of adenylate cyclase and the subsequent activation of cAMP-dependent protein kinases, ultimately leading to the inhibition of myometrial contraction (Kroeger & Marshall 1974; Riemer et al 1988; Roberts et al 1989). Clearly, side effects should be avoided with any treatment for premature delivery, but β_2-adrenoceptor stimulants may activate cardiac β_1-adrenoceptors to induce tachycardia at high doses. Therefore, the dose required for the efficacy of uterine relaxants should be lower than the dose exhibiting side effects. Application of β_2-adrenoceptor agonists with high potency, specificity and period of action would overcome the problem.

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Formoterol is a catecholamine analogue used for asthma therapy. Formoterol possesses potent β_2-adrenoceptor agonist effects and has been shown to have high potency and long-lasting action in animal experiments (Ida 1976a; Decker et al 1982). The potency of formoterol on histamine-induced bronchoconstriction was found to be approximately 50-fold that of salbutamol in guinea-pigs (Ida 1976b). Clinical data indicated that formoterol caused significant bronchodilation over at least 12 h (Ramsdale et al 1990; Faulds et al 1991). Specific β_2-adrenoceptor agonists have become a popular group of drugs employed for countering premature delivery, although in their conventional form, such as ritodrine, they are relatively short acting. Ritodrine has been shown to reduce delivery within 48 h after treatment (King et al 1988), but there is little convincing evidence that any further prolongation of pregnancy can be obtained.

In this study we have investigated the inhibitory effects of formoterol compared with those of ritodrine in pregnant rats.

Materials and Methods

Drugs and chemicals

Formoterol fumarate was obtained from Yamanouchi Pharmaceutical Co., Ltd (Tokyo, Japan). Ritodrine was purchased from Sigma Chemical Co. (St Louis, MO). Chemical and biological agents used were oxytocin (Sigma Chemical Co., St Louis, MO), ethyl carbamate (Urethane; Kishida chemical Co., Ltd, Osaka, Japan), [^{125}I](−)iodopindolol (Life Science Products Inc., Boston, MA), (±)-CGP-12177 (Research Biochemicals Inc., Natick, MA) and isobutylmethylxanthine (Sigma Chemical Co., St Louis, MO). The composition of the Ringer solution was (in mM); NaCl 150·4, KCl 5·4, $CaCl_2$ 0·36, $MgCl_2$ 0·19, $NaHCO_3$ 4·76, KH_2PO_4 0·15, Na_2HPO_4 0·56, glucose 2·78. The composition of the Krebs-Ringer bicarbonate buffer was (in mM); KCl 4·6, $MgSO_4{\cdot}7H_2O$ 1·16, $NaH_2PO_4{\cdot}H_2O$ 1·16, $CaCl_2{\cdot}2H_2O$ 2·5, NaCl 115·5, $NaHCO_3$ 21·9, glucose 11·1.

Myometrial contraction

Wistar–Imamichi strain rats (Imamichi Institute for Animal Production, Ibaraki, Japan) at the 18th–19th day of gestation were stunned and killed by decapitation. The uteri were rapidly removed from the animals and longitudinal muscle strips (2-mm wide, 10-mm long) were cut and suspended in Ringer solution at 37°C, bubbled with a mixture of 95% O_2 and 5% CO_2 at pH 7·4 in 20- or 10-mL organ baths. The preparations were loaded with a 0·5-g weight and contractions were recorded isometrically on an ink-writing recorder through a forced replacement transducer. Muscle contraction was induced with oxytocin 0·01 munits mL^{-1}, and evaluated as a percentage of size before the addition of drugs. The total frequency over 10 min was determined and IC50 values (molar concentration of agonists producing 50% of maximal response) were calculated from cumulative concentration–response curves.

Uterine motility in anaesthetized rats

Wistar–Imamichi strain rats at the 18th–21st day of gestation were anaesthetized with urethane (1·2 g kg^{-1}, i.p.). A balloon connected to a polyethylene catheter was inserted into a uterine horn after the removal of one foetus. Uterine motility was measured through transducers with amplifiers and a recorder. Percentage inhibition of the frequency of uterine contraction was calculated as an index, with reference to the contractions occurring over a 5-min period before the administration of each drug.

Influence on the heart rate

Wistar–Imamichi strain rats on the 18th–21st days of gestation were anaesthetized with urethane (1·2 g kg^{-1}, i.p.) and fixed in a supine position. Polyethylene catheters were introduced into their femoral arteries and veins, and connected to a pressure transducer with an amplifier and a recorder. The heart rate was derived from blood pressure signals using the amplifier. Formoterol, ritodrine or saline was introduced through a femoral vein catheter. Heart rates showed peak change for 5 min after administration (peak tachycardiac effects were seen 2–4 min after administration).

Radioligand binding study

Experiments were performed on three Wistar–Imamichi strain rats at the 19th day of gestation. The rats were killed by decapitation under ether anaesthesia and each uterus removed rapidly. Tissue was immediately placed in ice-cold saline solution, minced with scissors, and homogenized in 10 mL preparation buffer (sucrose 250 mM, Tris HCl 5 mM, EDTA 1 mM; pH 7·4) with a polytrone (1 × 2-s setting 7, 2 × 20-s setting 3·5). The homogenate was centrifuged for 15 min at 600 *g* and the supernatant was centrifuged for 15 min at 10 000 *g*. The pellet was discarded and the supernatant was centrifuged for 20 min at 50 000 *g*. The final pellet was resuspended in incubation buffer (Na_2HPO_4 10 mM, NaH_2PO_4 10 mM, NaCl 154 mM; pH 7·4) to yield a membrane protein concentration of 100–150 mg mL^{-1}. The fractions were stored at −80°C until use. The protein concentration was determined using a protein assay kit.

For the determination of total β-adrenoceptor density, membrane preparations were incubated with six concentrations of [^{125}I](−)iodopindolol (12·5–400 pM in a total volume 250 mL) for 40 min at 25°C. Incubation was terminated by addition of 10 mL washing buffer (Tris HCl 10 mM and NaCl 154 mM; pH 7·4) and rapid vacuum filtration through Whatman GF/C glass fibre filters. Filters were washed with an addition of 10 mL wash buffer and the radioactivity retained on filters was counted with a gamma counter. Non-specific binding was defined as radioactivity bound to membranes that was not displaced by a high concentration (1 μM) of the non-selective β-adrenoceptor antagonist (±)-CGP-12177 (Staehelin et al 1983). Specific binding of iodopindolol was defined as total binding minus

non-specific binding. It usually amounted to 70% at 100 pM iodopindolol.

For competition experiments, displacement of binding of 100 pM iodopindolol was performed by adding increasing concentrations of formoterol or ritodrine to the membrane suspension. Specific binding was determined as described above. Binding assays were carried out in triplicate.

cAMP production

Blood samples were collected from male Wistar strain rats (250–280 g; Nihon SLC, Hamamatsu, Japan) under ether anaesthesia. Lymphocytes were separated by density flotation in lymphocyte separation medium and samples were incubated in 1 mL Krebs-Ringer bicarbonate buffer containing either formoterol or ritodrine. The phosphodiesterase inhibitor isobutylmethyl-xanthine (0·25 mM) was added to all incubation tubes to prevent degradation of the cAMP. Incubation was conducted for 3 min at 37°C, and then the tubes containing the cells were placed in an incubator at 90°C for 20 min. The tubes were centrifuged for 10 min at 2000 *g*, and the supernatant was removed and stored at −80°C until assessed for cAMP concentration with an enzyme-linked immunosorbent assay (Cayman Chemical, Ann Arbor, MI).

Statistical analysis

Results are expressed as means ± standard errors. Statistical significance was calculated using a one-way analysis of variance test followed by the Dunnett multiple comparison test.

Results

Myometrial contraction

Contraction of uteri isolated from pregnant rats was induced by addition of oxytocin (0·01 units mL^{-1}). Formoterol and ritodrine inhibited the amplitude and frequency of uterine contractions in a dose-dependent manner (Figure 1) with IC50 values of $3{\cdot}8 \times 10^{-10}$ and $4{\cdot}7 \times 10^{-7}$ M, respectively. Formoterol was approximately 1000-fold more potent than ritodrine and the concentration causing inhibition of uterine contractions was much lower.

Uterine motility in anaesthetized rats

Figures 2 and 3 show data for the effects of formoterol and ritodrine on uterine motility when administered intravenously to pregnant rats. In most rats uterine motility was observed to occur spontaneously. Intravenous administration of formoterol (0·03–3 $\mu g\,kg^{-1}$) or ritodrine (30–3000 $\mu g\,kg^{-1}$) inhibited the uterine motility in a dose-dependent manner with ED50 values of 1·70 and over 3000 $\mu g\,kg^{-1}$, respectively.

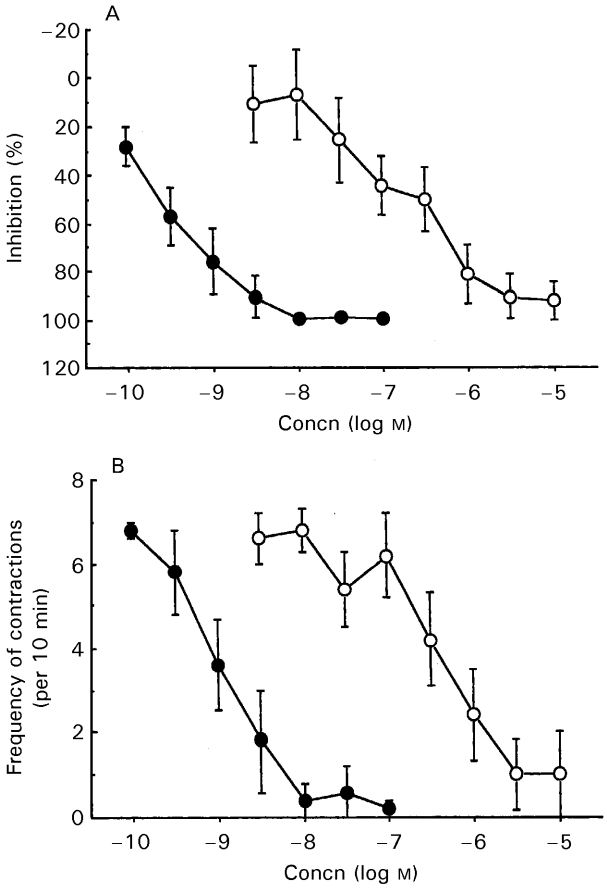

Figure 1. Effects of formoterol (●) and ritodrine (○) on oxytocin (10^{-2} munits mL^{-1})-induced contractile responses of isolated uteri of pregnant rat (18th–19th days of gestation). Each point and vertical bar represents the mean ± s.e.m. for five experiments. Data are for amplitude (A) and frequency (B) of uterine contraction.

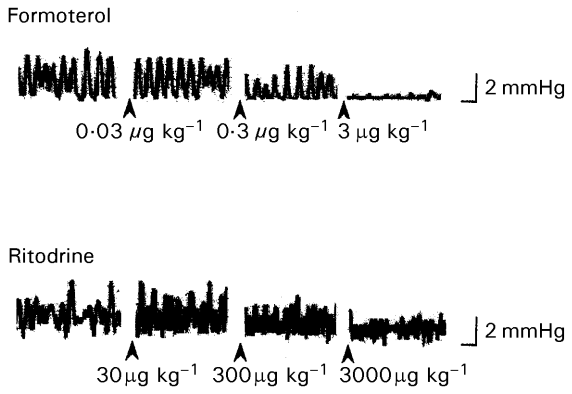

Figure 2. Effects of formoterol and ritodrine on uterine activity in pregnant rats.

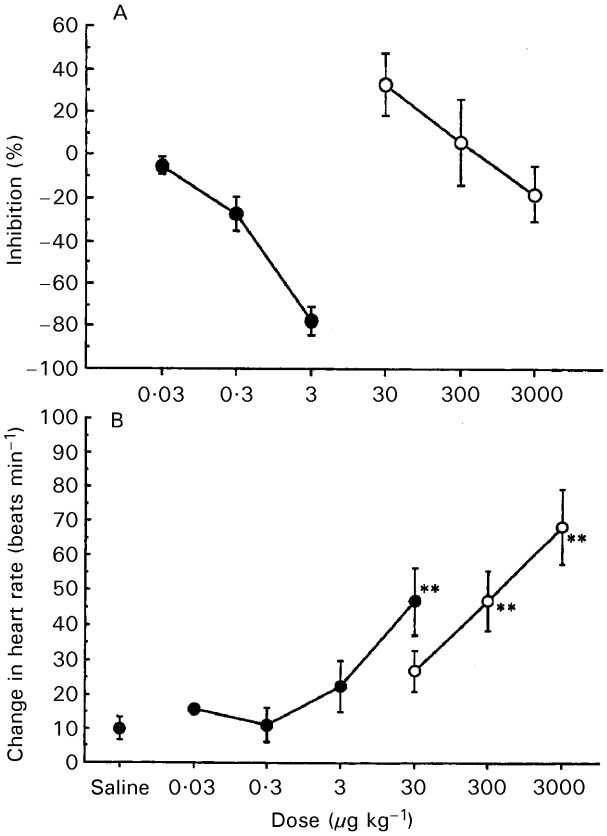

Figure 3. Effects of formoterol (●) and ritodrine (○) on uterine activity (A) and heart rate (B) in pregnant rats (18th–20th day of gestation). Each point and vertical bar represents the mean ± s.e.m. for five rats. The drugs tested were administered intravenously. $^{**}P < 0.01$ compared with control (Dunnett's test).

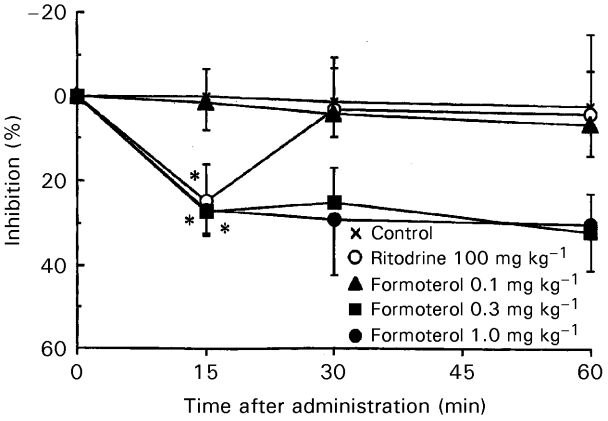

Figure 4. Effects of formoterol on uterine activity in pregnant rats (18th–21st day of gestation). Each point and vertical bar represents the mean ± s.e.m. for six or seven rats. The drug was administered orally. $^{*}P < 0.05$ compared with control (Dunnett's test).

Figure 4 shows data for the effects of formoterol and ritodrine on uterine motility when administered orally to pregnant rats. No significant inhibition was found with oral administration of saline. Formoterol (0·3 and 1 mg kg^{-1}, p.o.) inhibited uterine motility 15 min after administration, and this tended to persist for at least a 60-min period. Oral administration of ritodrine (100 mg kg^{-1}) inhibited motility 15 min after administration, but inhibitory effects were no longer seen at 30 or 60 min after administration.

Influence on the heart rate

Figure 3B shows the influence of formoterol and ritodrine on heart rate. Formoterol (0·03–30 μg kg^{-1}) and ritodrine (30–3000 μg kg^{-1}) increased heart rates of pregnant rats in a dose-dependent manner. The increase in heart rate with 30 μg kg^{-1} formoterol or 300 or 3000 μg kg^{-1} ritodrine was significant when compared with the saline-treated group.

Radioligand binding study

Binding of the non-selective β-receptor antagonist iodopindolol was saturable with high affinity (Figure 5). Scatchard analysis of the data resulted in a linear curve, indicating a single class of binding sites (Figure 5, insert). The calculated K_d and B_{max} values for iodopindolol were 720 pM and 83·2 fmol (mg protein)$^{-1}$, respectively, in the myometrial membranes of pregnant rats.

Formoterol and ritodrine inhibited iodopindolol binding to the myometrial membranes of pregnant

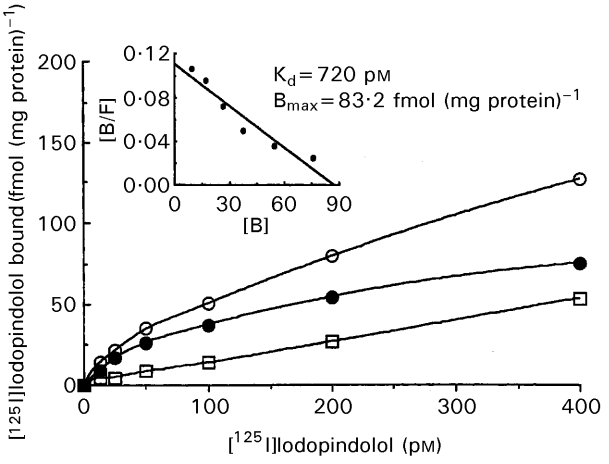

Figure 5. Binding of [^{125}I]iodopindolol to rat myometrial membranes as a function of increasing concentration. Binding was carried out as described in the methods with six concentrations of iodopindolol (12·5–400 pM) in the absence (○) and presence (□) of 1 mM (±)-CGP 12177 to determine specific binding (●). Insert: Scatchard plot of specific iodopindolol binding; B, specifically bound iodopindolol (fmol (mg protein)$^{-1}$); F, free iodopindolol (pM). The figure shows results of a typical saturation experiment.

rats with K_i values of 0·04 and 6·10 nM, respectively (Figure 6).

cAMP production

Table 1 shows the data for the effects of formoterol and ritodrine on cAMP production in rat lymphocytes. Lymphocytes incubated with formoterol or ritodrine was associated with a significant increase of cAMP in a dose-dependent manner. Significant increase of cAMP production was observed at 10^{-4} M ritodrine or formoterol, and the production of cAMP after the treatment of formoterol was higher than that of ritodrine.

Discussion

Premature delivery is a worldwide problem causing perinatal mortality and the possibility of neonates suffering from neurological or respiratory handicap. If delivery can be delayed by the use of agents, the risk may be much reduced (Creasy et al 1980; Schenken et al 1980; Papageorgiou et al 1989). Clinically, the β_2-adrenoceptor-agonist ritodrine is widely used as a tocolytic agent. On of its most striking effects is to reduce the incidence of delivery within 48 h after treatment. However, this immediate influence has not led to clinically important reductions in the rate of premature delivery or low birth weight (Moutquin et al 1992). Ritodrine stimulates a relatively specific β_2-adrenoceptor, although there is evidence that its potency is about 100–1000-fold lower than that of isoprenaline (Ikeda & Tamaoki 1984). Furthermore, almost all tocolytic agents, such as ritodrine, are short acting. A drug with greater and longer-acting relaxation effects might be beneficial for reducing the incidence of premature delivery. In this study, we have evaluated formoterol for this purpose.

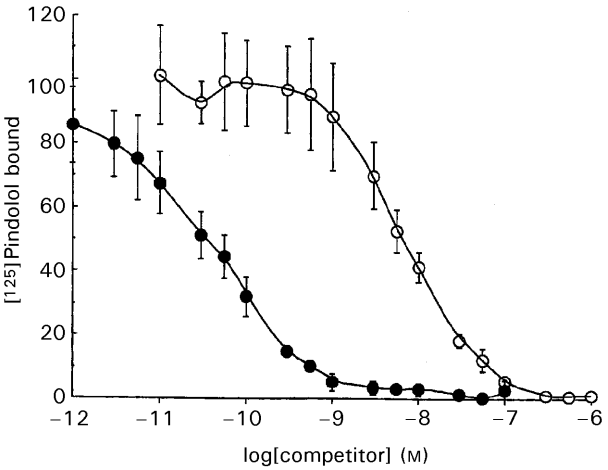

Figure 6. Competitive iodopindolol binding (100 pM) to rat myometrial membranes. Formoterol (●) and ritodrine (○) were used as competitors. The corresponding K_i values are indicated. Each point and vertical bar represents the mean ± s.e.m. of three experiments.

Table 1. Effects of ritodrine and formoterol on cAMP production in rat lymphocytes.

	Dose (M)	Cyclic AMP (fmol mL^{-1})
Control		75·4 ± 6
Ritodrine	10^{-6}	102·1 ± 10
	10^{-5}	103·9 ± 4
	10^{-4}	176·4 ± 7***
Formoterol	10^{-6}	163·8 ± 40
	10^{-5}	179·1 ± 15
	10^{-4}	296·4 ± 32***

Each value represents the mean ± s.e.m. for three experiments. ***$P < 0.001$ compared with control (Dunnett's test).

Formoterol and ritodrine completely inhibited uterine activity, and the pattern with ritodrine was similar to that reported by Ikeda & Tamaoki (1984), and the potency of formoterol was approximately 1000-fold greater. Furthermore, intravenously administered formoterol and ritodrine depressed uterine motility in a dose-dependent manner. Again the potency of formoterol was very much stronger, 1000–10000-fold that of ritodrine. In a previous study, orally administered formoterol was found to inhibit histamine-induced bronchoconstriction in guinea-pigs with an ED50 value (0·056 mg kg^{-1}) lower than those for salbutamol (2·38 mg kg-1) or isoprenaline (1·21 mg kg^{-1}) (Ida 1976b). This study indicated that formoterol exerted a potent relaxation influence on uterine smooth muscle as well as bronchi. In contrast with ritodrine, the inhibitory effects of formoterol tended to persist, at least for 60 min after administration. Pharmacokinetic properties of formoterol did not seem to exert a major influence in terms of the duration of inhibition of uterine contraction. The serum half-lives of formoterol and salbutamol, another β_2-adrenoceptor agonist, were similar, whereas only formoterol produced persistent bronchodilation (Löfdahl & Svedmyr 1987). Also, the β_2-adrenoceptor-agonist clenbuterol had a very long plasma half-life without causing prolonged bronchodilation (Yamamoto et al 1985). In a previous in-vitro study, it was confirmed that more lipophilic compounds, such as formoterol or salmeterol, had a longer duration of relaxant action on the guinea-pig trachea compared with the hydrophilic compounds salbutamol or fenoterol (Jeppsson et al 1989). Furthermore, based on radioligand-binding studies with (±)-[^{3}H]formoterol and on the resistance of (±)-[^{3}H]formoterol to

displacement by high concentrations of β-adrenoceptor agonists or antagonists, it was postulated that the binding stability of formoterol to the adrenoceptor may contribute to its long-lasting action (Lemoine 1992). The lipophilic compounds formoterol and salmeterol may produce long-lasting action by interaction with receptor membrane lipid layers.

In this study, it was demonstrated that formoterol had a higher efficacy for uterine relaxation compared with ritodrine. The dose required for the inhibition of uterine contraction was lower than that causing an increase in heart rate. It has been shown that formoterol is 60-times more selective for the β_2-subtype compared with the β_1-subtype in a binding study (Roux et al 1996). The selectivity may be related to suppression of uterine contraction without causing tachycardia.

We carried out β-adrenoceptor binding and cAMP production tests to examine the effects of formoterol and ritodrine in detail. In the radioligand binding study, the K_i values of formoterol and ritodrine were 0·04 and 6·10 nM, respectively, so that formoterol has approximately 150-fold the binding affinity of ritodrine. Furthermore, the maximum cAMP production of lymphocytes incubated with formoterol (296 ± 32 fmol mL^{-1}) was greater than that with ritodrine (176 ± 7 fmol mL^{-1}). However, the minimum concentration required to produce uterine relaxation was lower than that required to induce an increase in the amount of cAMP in lymphocytes. A significant positive correlation between lymphocyte and myometrial β-adrenoceptor densities has been reported (Michel et al 1989). Determination of β-adrenoceptor function in circulating lymphocytes may be a useful model to monitor myometrial β-adrenoceptor change or responses. However, different responses may exist between lymphocyte and myometrial β_2-adrenoceptors. Moreover, β-adrenoceptor agonists may not act solely through the cAMP system, which activates protein kinase A. The latter phosphorylates several proteins in the cell, effecting relaxation of smooth muscle. Protein kinase A also inhibits myosin light chain phosphorylation and phosphoinositide hydrolysis, while promoting Ca^{2+}/Na^+ exchange and Na^+/K^+ ATPase (Gerthoffer 1986; Gunst & Stropp 1988; Hall & Hill 1988; Madison & Brown 1988; Twort & van Breemen 1989). These effects are only observed at a relatively high concentration of β-adrenoceptor agonists. One important effect is the opening of membrane K^+ channels, and charybodotoxin, a selective blocker of conductance Ca^{2+}-activated K^+ channels, inhibits the bronchodilation due to β-adrenoceptor agonists (Jones et al 1990). Since such effects are observed at low concentrations of β-adrenoceptor agonists, this may be the major mechanism underlying smooth muscle relaxation. Furthermore, Kume et al (1992) suggested a potentially important functional pathway by which β-adrenoceptor stimulation activates a single K^+ channel, independent of cAMP dependent protein phosphorylation. The available data thus indicate that relaxation of uterine smooth muscle may occur independently of a rise in intracellular cAMP, and explains why there is a discrepancy between the low concentration needed to relax smooth muscle and the high concentrations necessary to elevate cAMP concentrations. The relaxation effects of formoterol or ritodrine may be due to K^+-channel activation by cAMP-dependent protein kinase and direct activation of a signal K^+ channel.

In summary, the inhibitory action of formoterol on pregnant rat uterus contraction was found to persist for longer and be greater than that of ritodrine. The potency of formoterol appears related to the high affinity for the β-adrenoceptor and the ability to produce intracellular cAMP. The results indicate that examination of formoterol treatment for the reduction in the incidence of premature delivery in clinical cases is warranted.

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