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Effects of mibefradil on uterine contractility

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Abstract

Mibefradil, a benzimidazolyl tetralol derivative, is a new Ca^{2+} channel antagonist which is structurally distinct from other Ca^{2+} channel antagonists such as nifedipine, verapamil and diltiazem. It is a very effective antihypertensive agent that is thought to achieve its action via a higher affinity block for low-voltage activated (T) than for high-voltage-activated (L) Ca^{2+} channels. Nevertheless, it blocks L-type Ca^{2+} channels in several tissues. In the present study, the effects of mibefradil on spontaneous rhythmic contractions and on contractions elicited by $CaCl_2$ (K^+-depolarized preparations) and oxytocin (in low Ca^{2+}/Ca^{2+}-free solutions) were investigated on uterus strips from pregnant and non-pregnant rats. Mibefradil (10^{-8}–3×10^{-6} M) caused concentration-dependent inhibition of spontaneous contractions of uterus strips from pregnant and non-pregnant rats with the IC_{50} values of 8.83×10^{-7} M; 5.94×10^{-7} M (amplitude) and 1.03×10^{-6} M; 5.48×10^{-7} M (frequency), respectively. Mibefradil (3 μM) caused a rightward shift in the concentration–response curves for $CaCl_2$ in K^+ (40 mM)-depolarized uterus strips taken from both pregnant and non-pregnant rats. Mibefradil (3 μM) was, however, more potent for antagonising $CaCl_2$ responses in uterus strips obtained from pregnant rats than in those from non-pregnant rats. Mibefradil (3 μM) had no effect on oxytocin-induced contraction in Ca^{2+}-free physiological salt solution (PSS) on uterus strips from non-pregnant rats. However, it markedly inhibited oxytocin-induced contraction of pregnant rat uterus strips in Ca^{2+}-free PSS. Thus, mibefradil probably antagonizes L-type Ca^{2+} channels as well as interferes with the intracellular Ca^{2+} release mechanism, which would be helpful in the development of a tocolytic agent.

Keywords: Uterus; Ca^{2+} channel; Mibefradil

1. Introduction

Mibefradil (Ro 40-5967) {1*S*, 2*S*)-2-[2[[3-(2-benzimidazolylpropyl] methylamino] ethyl]-6-fluoro-1,2,3,4-tetrahydro-1-isopropyl-2-napthylmethoxyacetate dihydrochloride}, a benzimidazolyl tetraline derivative, is a new Ca^{2+} channel antagonist which is structurally different from several classical Ca^{2+} channel antagonists such as nifedipine, verapamil and diltiazem. It differs from other Ca^{2+} channel blockers by its ability to potently block-T-type Ca^{2+} channels (Jimenez et al., 2000). There have been many studies on the cardiovascular effects of mibefradil since its promising antihypertensive and coronary vasodilatation activities were reported by Clozel et al. (1991). In addition to its inhibitory effect on contractility, mibefradil also facilitates the effect of endothelium-derived nitric oxide (NO) in vascular smooth muscle of porcine epicardial coronary arteries (Kung et al., 1995). Liu et al. (1999) have shown that mibefradil, an inhibitor of T-type Ca^{2+} current, blocked ionic currents other than Ca^{2+} currents, like the K^+ current expressed in fusion-competent myoblasts, and interfered with myoblast fusion. T-type channel blockade by mibefradil reduces blood pressure without stimulation of the renin–angiotensin system and thereby prevents damage to glomerular epithelial cells (Karam et al., 1999).

Most early studies showed that mibefradil effectively blocked L-type Ca^{2+} channels in the resting state in vascular smooth muscle cells (Mishra and Hermsmeyer, 1994a), whereas Aczel et al. (1998) suggested that mibefradil binds to the open state of α_{1A} channels expressed in Xenopus oocytes. Recently, Martin et al. (2000) showed that mibefradil has higher affinity for the inactivated state of the cloned T-channel isoforms α_{1G}, α_{1H} and α_{1I} than of a cloned L-type channel, α_{1C}.

Ion channel currents have been very well characterized in rat and human myometrium. For instance, rat myometrium is reported to have both slow L-type Ca^{2+} channels and fast Na^+ channels, but no T-type Ca^{2+} channels (Ohya and Sperelakis, 1989), while pregnant human myometrium is known to express both T- and L-type Ca^{2+} channels (Young et al., 1993). Investigations have been conducted to deter-

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mine whether ion channels change in their type or expression with gestation. A gradual increase in voltage-dependent Ca^{2+} channel (VDCC) α_1 subunit mRNA levels during the later half of gestation and a rapid increase in the β_1 subunit just before labor in rats (Tezuka et al., 1995) may produce changes that facilitate parturition. In contrast, little or no change in Ca^{2+} channel density in human myometrium throughout pregnancy was reported by Thornton et al. (2000). Several Ca^{2+} channel blockers have been shown to be potent inhibitors of spontaneous tension development by isolated uterine smooth muscles of rat and human (Granger et al., 1985; Hollingsworth et al., 1987).

It is essential for the well being of the fetus and the generation of rhythmic contractions in labour that myometrium should be able to relax fully during each contraction–relaxation cycle. Results of studies in rats suggest that Na^{+}–Ca^{2+} exchange is more important for relaxation in spontaneously contracting myometrium from pregnant rats than in uterus from non-pregnant rats (Taggart and Wray, 1997). Since mechanical activity of the uterus is dependent on the presence of extracellular Ca^{2+} (Bolton, 1979), it is predictable that tension development in the uterus should be inhibited by Ca^{2+} channel blockers. Assuming that tocolysis is beneficial, however, as there is no report on the effect of mibefradil on myometrial contractility, the present study was undertaken to examine whether mibefradil has a relaxant effect on uterus taken from pregnant and non-pregnant rats and whether this effect is related to Ca^{2+} translocation.

2. Materials and methods

2.1. Oestrogenization of rats

Virgin female Wistar rats (180–200 g), obtained from the Laboratory Animal Resource Section, Indian Veterinary Research Institute, Izatnagar (UP) were used in the present study, as per the Institutional Policy on Animal Use. The rats were given diethyl stilboestrol (1.5 mg/kg, i.p.) consecutively for 2 days prior to killing for further experimentation, unless otherwise stated.

2.2. Induction of pregnancy

Two or three female rats were kept for mating with one mature male rat in separate cages. Pregnancy was determined by microscopic examination of vaginal smears for the presence of sperm, which was taken to indicate day 1 of pregnancy.

2.3. Tension experiments

The rats were killed by cervical dislocation and the uterine horns were isolated and placed in cold oxygenated modified Ringer–Locke physiological salt solution (PSS) of the following composition (mM); NaCl, 136.9; KCl, 5.6; $NaHCO_3$, 11.9; $CaCl_2.2H_2O$, 2.2 and glucose, 5.6. The longitudinal strips, approximately 3×10 mm from each mid-horn region, were dissected out and suspended in an organ bath containing 20 ml PSS, bubbled continuously with O_2 at 37 ± 0.5 °C under a resting tension of 1*g* and equilibrated for a period of 1 h. Isometric contractions were recorded by a force transducer connected to an ink-writing oscillograph (Recorders and Medicare, India).

2.4. Experimental protocol

2.4.1. Effect of mibefradil on spontaneous contractions of rat uterus

After equilibration of the rat uterus strips for 60 min in PSS, spontaneous rhythmic contractions were recorded for a period of 15 min and then mibefradil was added cumulatively. A contact period of 15 min was maintained at each concentration. Both amplitude and frequency of rhythmic contractions were analyzed to study the effect of mibefradil.

2.4.2. $CaCl_2$-induced contractions of K^+ (40 mM)-depolarized rat uterus in the presence and in the absence of mibefradil

In order to study the modulation of voltage-dependent L-type Ca^{2+} channels by mibefradil, the effect of the Ca^{2+} channel blocker was studied on $CaCl_2$-induced contractions of K^+ (40 mM)-depolarized uterus strips, using the following protocol. The tissues were initially exposed to Ca^{2+}-free, EGTA (0.1 mM) containing PSS for 45 min during which the solution was changed every 15 min. Then, the tissues were further exposed to Ca^{2+}-free high-K^+ PSS (containing no EGTA) with repeated washes and left for 30 min. Concentration–response curves for Ca^{2+} were obtained by adding $CaCl_2$ (10^{-5}–10^{-2} M) in increments of 0.5 log units. Consecutive cumulative application of $CaCl_2$ was repeated after a 60-min recovery period. Then the tissues were incubated with mibefradil (3 μM) for 15 min prior to application of $CaCl_2$. The contractile responses to Ca^2 were expressed as percent maximum response (set as 100%) elicited by $CaCl_2$ (10^{-2} M) in the absence of any blocker. Some experiments were done with the prototype L-type Ca^{2+} channel blocker, nifedipine, for comparison.

2.4.3. Oxytocin-induced contractions in low Ca^{2+} (De Jalon) and Ca^{2+}-free PSS in the absence and in the presence of mibefradil (3 μM)

Some experiments were conducted in De Jalon solution (mM; NaCl, 154.0; KCl, 5.60; $CaCl_2.2H_2O$, 0.3; Dextrose, 5.50; $NaHCO_3$, 6.0, $MgCl_2.6H_2O$, 1.4; pH, 7.4) to study the effect of mibefradil on oxytocin-induced concentration-related contractile responses of uterus strips taken from oestrogenized non-pregnant and 19-day pregnant rats. It is well established that, in the absence of extracellular Ca^{2+}, oxytocin induces contraction of the myometrium through intracellular Ca^{2+} release (Luckas et al., 1999). In order to

study whether mibefradil had such an effect on intracellular Ca^{2+} release, the following experimental procedure was followed. The uterus strips were initially equilibrated in PSS followed by Ca^{2+}-free, EGTA (2 mM) containing PSS for a period of 30 min. Then, the tissues were further exposed to Ca^{2+}-free PSS (containing no EGTA) with repeated washes and left for 30 min. Then the strips were contracted with a submaximal concentration of oxytocin (3×10^{-2} IU/ml). After obtaining two consecutive contractions at an interval of 30 min, the uterus strips were incubated with mibefradil (3 μM) for 15 min and again the contractile response to oxytocin was recorded.

2.5. Statistics

Results are given as means ± S.E.M. Student's *t*-test was employed to test for significance at the level of $P < 0.05$. IC_{50}/EC_{50} values were calculated by regression analysis (Snedecor and Cochran, 1980). The concentration ratio of $CaCl_2$ was calculated by the formula: EC_{50} in the presence of Ca^{2+} channel antagonist/EC_{50} in the absence of Ca^{2+} channel antagonist.

3. Results

3.1. Effect of mibefradil on spontaneous contractions of rat uterus

The uterus strips from non-pregnant rats exhibited spontaneous rhythmic contractions in PSS, with a mean amplitude of 1.48 ± 0.17*g* and mean frequency of 1.02 ± 0.04 contractions/min ($n = 11$). The effect of mibefradil on spontaneous rhythmic contractions of uterus strips is shown in Fig. 1. Cumulative addition of mibefradil (10^{-8}–3×10^{-6} M) in increments of 0.5 log units, produced a concentration-dependent inhibition of amplitude and frequency of spontaneous contractions, with complete inhibition occurring at 3×10^{-6} M. The threshold concentration of mibefradil to produce an inhibitory effect on amplitude and frequency was 3×10^{-8} M. The concentration–response curves for mibefradil are shown in Fig. 2. The mean IC_{50} values for mibefradil to inhibit amplitude and frequency were

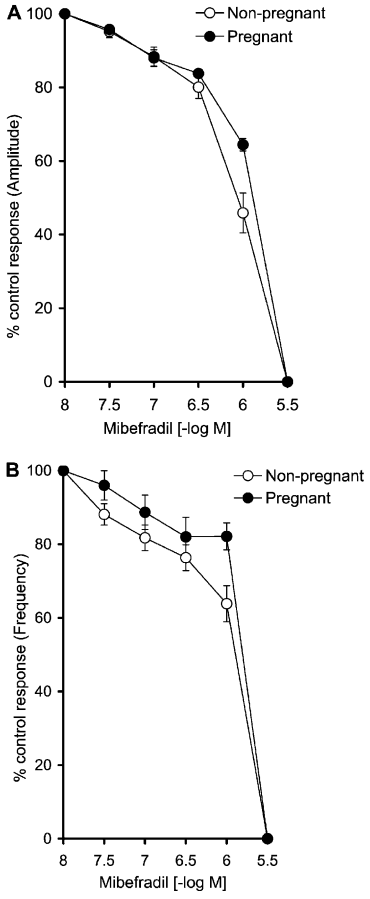

Fig. 2. Effect of mibefradil on the spontaneous rhythmic contractions of uterine strips taken from non-pregnant and pregnant rats. (A) Concentration-related reduction of the amplitude of rhythmic contractions by mibefradil (10^{-8}–3×10^{-6} M), added cumulatively at intervals of 0.5 log unit. (B) Concentration-related inhibition of the frequency of rhythmic contractions by mibefradil (10^{-8}–3×10^{-6}M).

5.94×10^{-7} M [CL $3.62 - 9.74 \times 10^{-7}$ M] and 5.48×10^{-7} M [CL $3.31 - 9.07 \times 10^{-7}$ M; $n = 11$], respectively.

Uterus strips from 18/19th-day pregnant rats exhibited spontaneous contractions in PSS. Mean isometrically developed tension and frequency were 1.87 ± 0.01*g*, 1.12 ± 0.12 contractions/min ($n = 5$), respectively. Neither the amplitude nor the frequency of rhythmic contractions

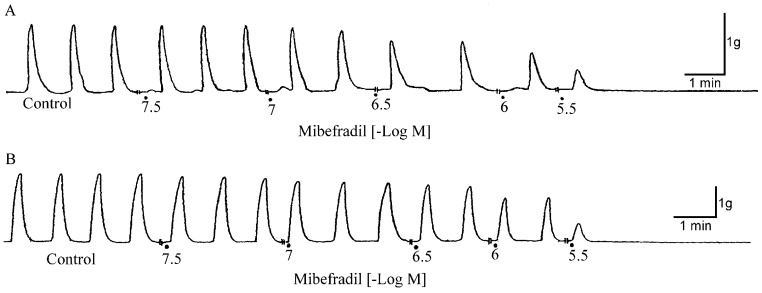

Fig. 1. Effect of mibefradil on the spontaneous rhythmic contractions of rat uterus. Representative tracings show the effect of mibefradil on uterine strips obtained from (A) non-pregnant and (B) pregnant rats.

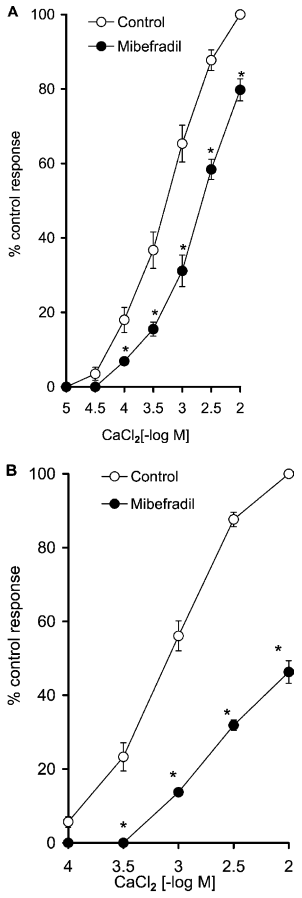

Fig. 3. Concentration–response curves for $CaCl_2$ in K^+ (40 mM)-depolarized rat uterus, in the presence and the absence of mibefradil (3 μM), uterus strips obtained from (A) non-pregnant rats ($n=6$), (B) pregnant rats ($n=6$); *$P<0.05$.

of strips from pregnant rats was significantly different from that of the strips from non-pregnant rats. Cumulative addition of mibefradil (10^{-8}–3×10^{-6} M) produced a concentration-related inhibitory effect on the amplitude and frequency of spontaneous contractions, with complete inhibition occurring at 3×10^{-6} M. The mean IC_{50} values for mibefradil inhibition of amplitude and frequency were 8.83×10^{-7} M [CL 8.47–9.19×10^{-7} M] and 1.03×10^{-6} M [CL 5.06×10^{-7}–2.09×10^{-6} M], respectively. The threshold concentration to inhibit amplitude and frequency was 3×10^{-8} (Fig. 2B).

As illustrated in Fig. 2, mibefradil (10^{-8}–3×10^{-6} M) produced a steep concentration–response curve for inhibition of the rhythmic contractions. Therefore, the effects of mibefradil at lower concentrations were not significantly different from each other.

3.2. Effects of mibefradil and nifedipine on $CaCl_2$-induced contractions of K^+ (40 mM)-depolarized rat uterus

$CaCl_2$ (10^{-5}–10^{-2} M) added cumulatively to K^+-depolarized uterus strips from non-pregnant rats produced a concentration-related contraction. The threshold concentration of $CaCl_2$ and the concentration required to produce a maximal contraction were 3×10^{-5} and 10^{-2} M, respectively. The mean E_{max} generated by $CaCl_2$ was $1.14\pm0.19g$ with EC_{50} of 5.06×10^{-4} M [CL 3.44–7.45×10^{-4} M]. Preincubation of the tissues with mibefradil (3 μM) produced a rightward shift of the concentration–response curve (Fig. 3A). The mean EC_{50} of $CaCl_2$ in the presence of mibefradil was 1.86×10^{-3} [CL 1.57–2.20×10^{-3} M] and the calculated mean concentration ratio was 4.06 ± 0.81 ($n=6$).

For pregnant rat uterus strips, the threshold concentration of $CaCl_2$ and concentration required to produce the maximal contraction were 10^{-4} and 10^{-2} M, respectively. The force generated at the highest concentration (E_{max}) of $CaCl_2$ was $1.77\pm0.25g$ with the EC_{50} value of 7.94×10^{-4} M [CL 6.42–9.81×10^{-4} M]. Pretreatment of uterus strips with mibefradil (3 μM) shifted the Ca^{2+}-concentration–response curve to the right and decreased the maximal response by about 52% ($n=6$; Fig. 3B).

Nifedipine (10^{-8} M) not only caused a rightward shift of the concentration–response curves for $CaCl_2$ with K^+-depolarized uterus strips from non-pregnant and pregnant rats, but also suppressed the maxima (Fig. 4).

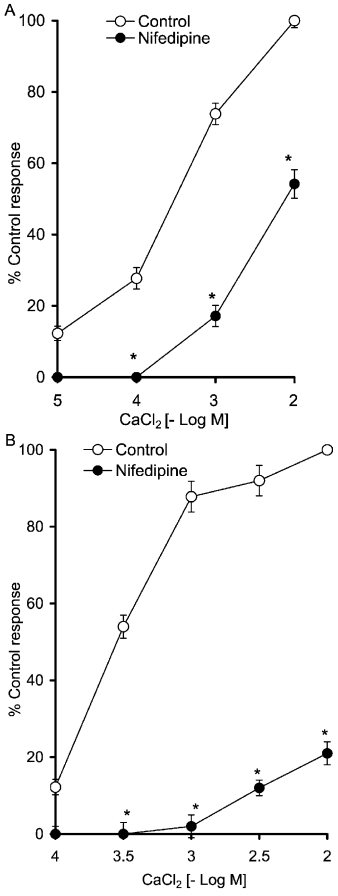

Fig. 4. Effects of nifedipine (10^{-8} M) on $CaCl_2$-induced contractions of K^+-depolarized uterus strips obtained from non-pregnant (A: $n=6$) and pregnant (B: $n=6$) rats; *$P<0.05$.

3.3. Effect of mibefradil (3 μM) on oxytocin-induced contractions of uterine strips in low-Ca^{2+} and Ca^{2+}-free PSS

Oxytocin, added cumulatively (10^{-5}–10^{-1} IU/ml), produced a concentration-related contractile response on uterus strips obtained from non-pregnant rats in De Jalon solution (containing 0.3 mM Ca^{2+}). The threshold concentration of oxytocin and concentration required to produce a maximal contraction were 10^{-5} and 10^{-1} IU, respectively. The mean E_{max} generated by oxytocin was $0.79 \pm 0.09g$ with the EC_{50} value of 1.95×10^{-4} IU [CL $1.01–3.78 \times 10^{-4}$ IU]. Preincubation of strips with mibefradil (3×10^{-6} M) produced a small righward shift with an EC_{50} of 8.07×10^{-4} IU [CL 2.65×10^{-4}–2.46×10^{-3} IU] ($n=6$; Fig. 5A). Fig. 5B depicts the effect of mibefradil (3×10^{-6} M) on oxytocin (10^{-5}–3×10^{-2} IU)-induced concentration-dependent contractions of the uterus strips taken from 19-day pregnant rats. In the absence of mibefradil, the maximal tension achieved with oxytocin (3×10^{-2} IU) was $1.73 \pm 0.47g$ ($n=6$). Pretreatment of the tissues with mibefradil (3×10^{-6} M) shifted the concentration–response curve for oxytocin to right and reduced the maxima by about 46%.

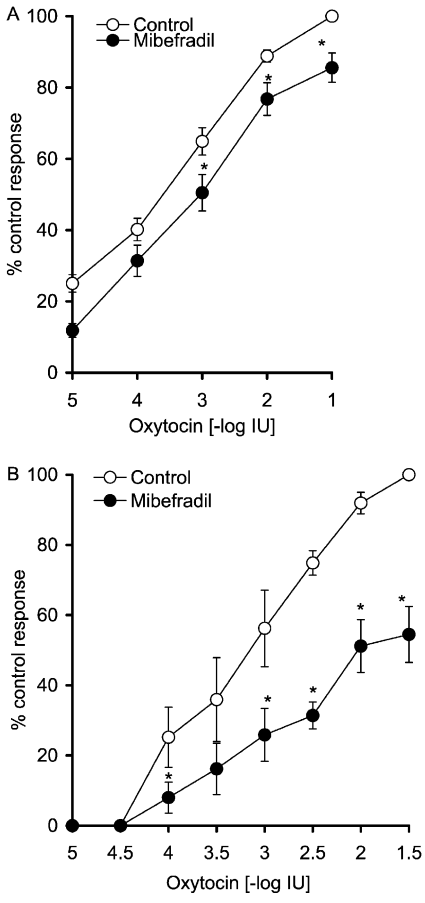

Fig. 5. Effect of mibefradial on oxytocin-induced contraction of non-pregnant (A) and pregnant (B) rat uterus strips bathed in low Ca^{2+} (0.3 mM) De Jalon solution ($n=6$); *$P<0.05$.

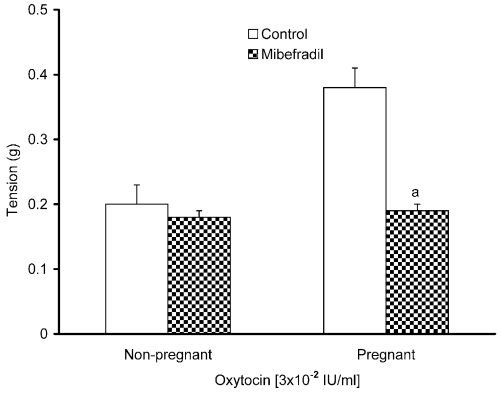

Fig. 6. Oxytocin (3×10^{-2} IU/ml)-induced contractions of rat uterus strips in the presence and in the absence of mibefradil (3 μM). a: $P<0.001$ as compared to controls from pregnant rats.

Oxytocin induced a measurable contractile response of uterus strips in the absence of external Ca^{2+}. The mean isometrically developed tension with oxytocin (3×10^{-2} IU/ml) was found to be $0.20 \pm 0.03g$ ($n=10$). Preincubation of tissues with mibefradil (3 μM) reduced the force generated with oxytocin to $0.18 \pm 0.03g$, which was not significantly different from the control (Fig. 6).

Uterus from pregnant rats was found to be highly sensitive to the spasmogenic action of oxytocin (3×10^{-2} IU/ml) and the force generated was $0.38 \pm 0.01g$. Preincubation of strips with mibefradil (3 μM), resulted in a highly significant ($P<0.001$) reduction in the force $0.19 \pm 0.01g$ developed with oxytocin (Fig. 6).

4. Discussion

Spontaneous contractility of rat uterus is primarily dependent on extracellular Ca^{2+} (Bolton, 1979) and several Ca^{2+} channel blockers, including nifedipine, are potent inhibitors of spontaneous contractions. Voltage-gated L-type Ca^{2+} channels have been very well characterized in rat uterus (Jmari et al., 1986; Amedee et al., 1987) and they have been reported to play a critical role in the generation of action potentials as well as in the regulation of myometrial contractility. The observation that mibefradil inhibits the spontaneous contractions of uterus obtained both from pregnant and non-pregnant rats perhaps relates to the inhibition of L-type Ca^{2+} channels in this tissue. Mishra and Hermsmeyer (1994b) reported that, in vascular smooth muscle cells, mibefradil not only blocks T-type Ca^{2+} channels more selectively but also blocks L-type Ca^{2+} channels. Considering the absence of T-type Ca^{2+} channels either in pregnant or non-pregnant rat myometrium (Ohya and Sperelakis, 1989), it is believed that the inhibition of spontaneous rhythmic contractions by mibefradil results from the antagonism of L-type Ca^{2+} channels. The antag-

onism of voltage-gated L-type Ca^{2+} channels is further substantiated by the observation that mibefradil caused a rightward shift in the concentration–response curves for $CaCl_2$ in K^+-depolarized uterine strips. It is well known that the $CaCl_2$-induced contraction in K^+-depolarized smooth muscle preparations results from an inward movement of Ca^{2+} through voltage-dependent L-type Ca^{2+} channels (Edwards et al., 1986). The involvement of L-type Ca^{2+} channels in mibefradil-induced inhibition of $CaCl_2$ responses in K^+-depolarized tissues is further substantiated by the observation that nifedipine, a prototype calcium channel blocker, also potently inhibits Ca^{2+}-induced contraction.

Oxytocin binds to a G-protein coupled receptor expressed in myometrium (Kimura et al., 1992). Activation of the oxytocin receptor stimulates G-proteins of $G_{\alpha q/11}$ family, resulting in an increase in phosphatidylinositol (PI) turnover and an increase in $[Ca^{2+}]_i$ (Sanborn et al., 1998). Besides block of voltage-dependent Ca^{2+} channels, mibefradil has been reported to inhibit Ca^{2+} release from sarcoplasmic reticulum (SR) by agonists such as norepinephrine in vascular smooth muscle cells (Mishra and Hermsmeyer, 1994c). It was, therefore, of interest to examine if mibefradil has a similar mechanism of action on the myometrium to inhibit contractility. Oxytocin is known to release intracellular Ca^{2+} to cause force production in myometrium in the absence of extracellular Ca^{2+} (Luckas et al., 1999). Besides the intracellular Ca^{2+} release mechanism, L-type Ca^{2+} channels also significantly contribute to the oxytocin-induced increase in intracellular $[Ca^{2+}]$ (Sanborn, 2001).

In non-pregnant rat uterus, it was observed that mibefradil caused a rightward shift of the concentration–response curve elicited by oxytocin in the presence of extracellular Ca^{2+}. This observation may relate to the antagonism of L-type Ca^{2+} channels by mibefradil. However, when extracellular Ca^{2+} was removed from the medium, mibefradil had no effect on the force generation by oxytocin in non-pregnant rat uterus. On the contrary, there was a significant reduction in the oxytocin-induced contractions of pregnant rat myometrium in the presence of mibefradil, thereby suggesting an effect of the Ca^{2+} channel blocker on intracellular Ca^{2+} release by the agonist. It is difficult, however, at the present stage, to explain the different mechanism of action of mibefradil on pregnant and non-pregnant rat myometrium. Nevertheless, it is believed that mibefradil-induced inhibition of myometrial contractility in non-pregnant rats results from an action of the drug on voltage-dependent L-type Ca^{2+} channels. On the other hand, mibefradil inhibits myometrial contractility of the pregnant rats through block of voltage-dependent L-type Ca^{2+} channels as well as agonist-induced sarcoplasmic reticular Ca^{2+} release.

Based on the present observations, it is concluded that mibefradil has a tocolytic effect on rat uterus. However, in vivo studies are required to determine the therapeutic efficacy of mibefradil as a tocolytic agent. Further, while the tension experiments provide evidence of L-type Ca^{2+} channel antagonism as well as inhibition of intracellular Ca^{2+} release by mibefradil, direct evidence for the action of this drug can be obtained from studies on ion channel currents and intracellular Ca^{2+} measurements. Since mibefradil is a potent T-type Ca^{2+} channel blocker, further studies on pregnant human myometrium (reported to express T-type Ca^{2+} channels) will clarify the importance of T-type Ca^{2+} current in myometrial function.

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