Korean J Physiol Pharmacol
Vol 13: 241−249, June, 2009

Role of T-type Ca^{2+} Channels in the Spontaneous Phasic Contraction of Pregnant Rat Uterine Smooth Muscle

Si-Eun Lee, Duck-Sun Ahn, and Young-Ho Lee

Department of Physiology, BK 21 Project for Medical Sciences, College of Medicine, Yonsei University, Seoul 120-752, Korea

Although extracellular Ca^{2+} entry through the voltage-dependent Ca^{2+} channels plays an important role in the spontaneous phasic contractions of the pregnant rat myometrium, the role of the T-type Ca^{2+} channels has yet to be fully identified. The aim of this study was to investigate the role of the T-type Ca^{2+} channel in the spontaneous phasic contractions of the rat myometrium. Spontaneous phasic contractions and $[Ca^{2+}]_i$ were measured simultaneously in the longitudinal strips of female Sprague-Dawley rats late in their pregnancy (on day 18∼20 of gestation: term=22 days). The expression of T-type Ca^{2+} channel mRNAs or protein levels was measured. Cumulative addition of low concentrations (< 1 μM) of nifedipine, a L-type Ca^{2+} channel blocker, produced a decrease in the amplitude of the spontaneous Ca^{2+} transients and contractions with no significant change in frequency. The mRNAs and proteins encoding two subunits (α1G, α1H) of the T-type Ca^{2+} channels were expressed in longitudinal muscle layer of rat myometrium. Cumulative addition of mibefradil, NNC 55-0396 or nickel induced a concentration-dependent inhibition of the amplitude and frequency of the spontaneous Ca^{2+} transients and contractions. Mibefradil, NNC 55-0396 or nickel also attenuated the slope of rising phase of spontaneous Ca^{2+} transients consistent with the reduction of the frequency. It is concluded that T-type Ca^{2+} channels are expressed in the pregnant rat myometrium and may play a key role for the regulation of the frequency of spontaneous phasic contractions.

Key Words: Calcium channels, Nickel, Mibefradil, NNC 55-0396, Spontaneous contractility

INTRODUCTION

The uterus maintains a sustained muscle tone to support the growing fetus without coordinated contractions during pregnancy (quiescence phase). At the end of gestation, it undergoes many changes regarding hormone activities, density and activity of ion channels, and of gap junctions, which result in rhythmic, forceful, and highly coordinated spontaneous contractions to labor (Riemer and Heymann, 1998; Challis et al., 2000; Parkington and Coleman, 2001).

It is well known that spontaneous phasic contraction of uterine smooth muscle - myometrium - is related to an increase in the concentration of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) and that voltage-dependent Ca^{2+} channels represent the major machinery for $[Ca^{2+}]_i$ elevation (Wray et al., 2003). Two types of Ca^{2+} channels, L (long-lasting)-type and T (transient)-type Ca^{2+} channel, have been described in the myometrium. L-type Ca^{2+} channel has been identified in myometrium by electrophysiological (Parkington and Coleman, 1988), pharmacologic (Chien et al., 1996; Collins et al., 1996), and molecular studies (Mershon, 1994). It is also known that Ca^{2+} entry during the action potential is via L-type Ca^{2+} channel which is opened by spontaneous pacemaker activity and is an essential component for excitation-contraction coupling in uterine smooth muscle (Riemer and Heymann, 1998; Parkington and Coleman, 2001). On the other hand, the presence and the functional significance of T-type Ca^{2+} channels in the myometrium are less well defined.

It has been previously demonstrated in electrophysiological studies that T-type Ca^{2+} channels are present in human (Young et al., 1993; Knock and Aaronson, 1999) myometrium but not evidenced in rat myometrium (Ohya and Sperelakis, 1989; Inoue and Sperelakis, 1991). It has been also demonstrated that the mRNAs of T-type Ca^{2+} channel are expressed in human myometrium (Blanks et al., 2007). However, in a recent molecular study on the rat, it was demonstrated that both Ca_v 3.1 (α1G) and Ca_v 3.2 (α1H), α-subunits of T-type Ca^{2+} channels, were expressed in circular and longitudinal layers of myometrium and that the relative expression profile of these channels differed, de-

Received May 27, 2009, Revised June 4, 2009,
Accepted June 19, 2009

Corresponding to: Young-Ho Lee, Department of Physiology, College of Medicine, Yonsei University, C.P.O. Box 8044, Seoul 120-752, Korea. (Tel) 82-2-2228-1708, (Fax) 82-2-393-0203, (E-mail) yhlee@yumc.yonsei.ac.kr

ABBREVIATIONS: $[Ca^{2+}]_i$, concentration of intracellular free Ca^{2+}; Fura-2/AM, acetoxymethyl ester of Fura-2; HEPES, [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid]; dNTP, deoxynucleoside triphosphate; RT, reverse transcriptase; PCR, polymerase chain reaction; ECL, enhanced chemiluminescence; NNC 55-0396, ([(1S, 2S)-2-(2-(*N*-[(3-Benzimidazol-2-yl)propyl]-*N*-methylamino)ethyl)-6-fluoro-1,2,3,4-tetrahydro-1-isopropyl-2-naphtyl cyclopropanecarboxylate dihydrochloride]; ANOVA, analysis of variance; L-type Ca^{2+} channel, long-lasting-type Ca^{2+} channel; T-type Ca^{2+} channel, transient-type Ca^{2+} channel.

pendent on gestational age and layer (Ohkubo et al., 2005).

As the T-type Ca^{2+} channel may respond to the pacemaker potential and depolarize the plasma membrane sufficiently to allow for activation of other voltage-dependent ion channels such as L-type Ca^{2+} channels, elucidation of the role of T-type Ca^{2+} channels in spontaneous contractions may provide important clues to the nature of the molecular mechanism responsible for the generation of spontaneous contractions. In a recent study, it was demonstrated that treatment of nickel, a T-type Ca^{2+} channel inhibitor, reduced frequency without changing the force of spontaneous contractions in the human myometrium (Blanks et al., 2007). This indeed suggests that the T-type Ca^{2+} channels may be involved in the initiation of action potentials in myometrium, but the functional significance is not fully understood.

The aim of the present study was to investigate whether the T-type Ca^{2+} channels are present in rat myometrium and what the role of the T-type Ca^{2+} channels is in the spontaneous phasic contractions of the rat myometrium.

METHODS

The investigation conforms with the *Guide for the Care and use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Simultaneous measurement of $[Ca^{2+}]_i$ and force

Female Sprague-Dawley rats in their late pregnancy (on 18～20 days) were killed by cervical dislocation. All procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee. The uterine horns were isolated and immediately placed in an ice-cold, oxygenated normal Tyrode solution composed of (in mmol/l): Glucose 12; NaCl 135; KCl 5.4; $MgCl_2$ 1.2; HEPES 10; $CaCl_2$ 2.5. Blood, placental tissue, endometrium and the circular smooth muscle layer were gently removed and longitudinal myometrial strips, approximately 1.5×3 mm from each horn, were dissected out with a fine scissor under a binocular microscope. One end of the tissue strip was tied by a thin thread to connect to the transducer.

$[Ca^{2+}]_i$ was measured according to the method described by Yeon et al. (2002) using fluorescent Ca^{2+} indicator, Fura-2. The longitudinal strips were exposed to acetoxymethyl ester of Fura-2 (Fura-2/AM, 5 μM) and 0.02% cremophor EL in normal Tyrode solution for 3～4 hr at room temperature. At the end of the loading period, the muscle strips were washed with normal Tyrode solution for 30 min to remove extracellular Fura-2/AM and were held horizontally in a temperature-controlled 5 ml organ chamber. The normal Tyrode solution was maintained at 37°C and was continuously aerated with 100% O_2. After 30 min of washing in normal Tyrode solution, one end of the muscle strip was connected to force-displacement transducer (Harvard, Holliston, MA, USA) to monitor the muscle contraction. Muscle strips were stretched passively to the optimal length by imposing a stretch of 140% of resting length and equilibrated for 60 min. Muscle strips were illuminated alternately (48 Hz) at two excitation wavelengths (340 and 380 nm). The intensity of 500 nm fluorescence (F_{340} and F_{380}) was measured by using a fluorimeter (CAF110, JASCO, Tokyo, Japan). The ratio of F_{340} to F_{380} (F_{340}/F_{380}) was calculated as an indicator of $[Ca^{2+}]_i$. After regular spontaneous phasic contractions had been established (0～60 min), several inhibitors were added to the strips to determine their effects on Ca^{2+} transient and force. In some experiments, 0-Ca^{2+} solution was used: normal Tyrode solution in which $CaCl_2$ had been omitted and 1 mM EGTA added.

Reverse transcription - polymerase chain reaction

For the isolation of total RNA, the dissected myometrial tissue was broken down using a pestle in 1 ml easy-BLUE™ (Intron Biotechnology, South Korea) and isolation was achieved by means of the manufacturer's instructions. RNA concentration was measured by ultraviolet absorbance at 260 nm using a spectrophotometer. First-strand complementary DNA was synthesized by incubating 2 μg of RNA at 42°C for 60 min in a final volume of 20 μl containing 5× RT buffer, 10 U/μl of AMV Reverse Transcriptase, 0.2 mM of oligo dT, 2.5 mM of deoxynucleoside triphosphate (dNTP) mixture, and 10 U/μl RNase inhibitor (Power cDNA Synthesis Kit, Intron Biotechnology, South Korea). Complementary DNA (2 μg) was amplified using primers for α1G and α1H in a final volume of 20 μl, containing 5 U/μl of Taq DNA polymerase (i-MAX™ DNA polymerase, Intron Biotechnology, South Korea), 2.5 mM of each dNTP, 10× PCR buffer, 20 pmol of α1G and α1H primers, and sufficient water. The PCR reaction mixtures were heated to 94°C for 5 min and amplified in 35 cycles. Each cycle consisted of denaturation at 94°C for 30 sec, annealing at 55.4°C for 30 sec, and extension at 72°C for 30 sec. The primers used were as follows: forward, 5'-gdaaagtctccaagcacatc-3'; reverse, 5'-ctgacagcaatggagtgtct-3' for the α1G subunit (with an expected PCR product of 262 base pairs); forward, 5'-ggacagtgaccaaagtgtga-3'; reverse, 5'-ccagctacaggctcatct-3' for the α1H subunit (with an expected PCR product of 218 base pairs). Mixtures were separated on a 1% agarose gel and after staining with ethidium bromide, PCR products were visualized under UV light.

Western blot

Longitudinal strips were dissected and quick-frozen in dry ice and homogenized in buffer containing Triton X 100 1 ml, NaCl 0.088 g, Tris base 0.012 g, NP40 50 μl, and water in final volume of 10 ml. Protein-matched samples (100 μg protein/lane) were subjected to electrophoresis on 6% SDS-polyacrylamide gels and then were transferred to nitrocellulose membranes. Reversible Ponceau staining of the membranes was performed to confirm the equal loading of protein. Membranes were incubated in 5% skim milk in PBS-Tween 20 buffer for 1 hr at room temperature and then were incubated for 2 hr at room temperature in the presence of primary antibodies to α1G (1 : 200; Alomone Labs, Jerusalem, Israel) and α1H (1 : 200; Alomone Labs, Jerusalem, Israel). Membranes were washed and then incubated with horseradish peroxidase conjugated secondary antibody (1 : 5,000; Calbiochem, Darmstadt, Germany) for 1 hr at room temperature. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL; Amersham, Uppsala, Sweden). Developed films from ECL were scanned.

Drugs and chemicals

The following drugs were used: nifedipine (Sigma, St Louis,

MO, USA), NNC 55-0396 ([(1S,2S)-2-(2-(*N*-[(3-Benzimidazol-2-yl)propyl]-*N*-methylamino)ethyl)-6-fluoro-1,2,3,4-tetrahydro-1-isopropyl-2-naphtyl cyclopropanecarboxylate dihydrochloride]) (Sigma, St Louis, MO, USA), mibefradil (Sigma, St Louis, MO, USA), nickel (Sigma, St Louis, MO, USA), Fura-2/AM (Molecular Probes, Eugene, OR, USA). General laboratory reagents were used analytical grade or better.

Statistics

Data are expressed as the mean±SEM and *n* indicates the number of strips. Force was expressed as a relative percentage of the amplitude of spontaneous phasic contractions or of the 70 mM K^+ solution. Differences between means tested using ANOVA. Significant differences were taken at the $p<0.05$ level.

RESULTS

Effect of removing external Ca^{2+} and nifedipine on spontaneous Ca^{2+} transients and contractions

The myometrial strips from pregnant rats exhibited spontaneous rhythmic Ca^{2+} transients and contractions in normal Tyrode solution, with a mean contractile amplitude of 12.16±2.30 mN and mean frequency of 0.69±1.14 contractions/min (n=10). Under control conditions, spontaneous Ca^{2+} transients and contractions of consistent amplitude and frequency could be recorded for several hours. The effect of removing external Ca^{2+} (0-Ca^{2+} solution) or 1 μM nifedipine, a blocker of L-type Ca^{2+} channels, on the spontaneous Ca^{2+} transients and contractions of uterus strips is shown in Fig. 1. The spontaneous contractions stopped and $[Ca^{2+}]_i$ fell upon changing to 0-Ca^{2+} solution or adding 1 μM nifedipine (Fig. 1A, B).

Effect of low concentration of nifedipine on spontaneous Ca^{2+} transients and contractions

To determine the role of L-type Ca^{2+} channels on the frequency and amplitude of spontaneous Ca^{2+} transients and contractions, effect of low concentration of nifedipine, a blocker of L-type Ca^{2+} channels, was tested. As shown in Fig. 1C, D, cumulative addition of low concentrations, which did not completely abolished spontaneous contractions, produced a decrease in the amplitude of spontaneous Ca^{2+} transients and contractions. However, in contrast, the frequency of spontaneous Ca^{2+} transients and contractions was not significantly changed by these concentrations of nifedipine.

Expression of T-type Ca^{2+} channels in rat myometrium

Expression of the mRNAs and proteins encoding two subunits (α1G and α1H) of T-type Ca^{2+} channel was examined using comparative kinetic RT/PCR and western blot in longitudinal muscle layer. As shown in Fig. 2, two sub-

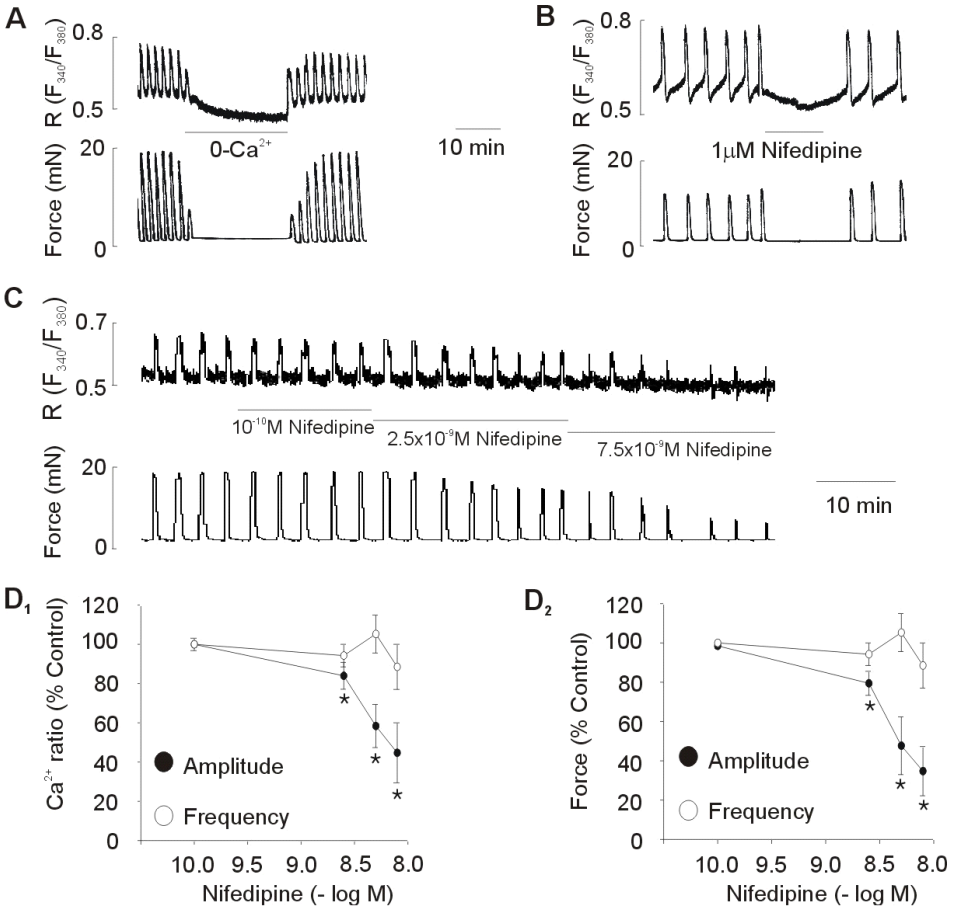

Fig. 1. Effect of removing external Ca^{2+} and nifedipine on spontaneous Ca^{2+} transients and contractions. (A, B) Effect of removing external Ca^{2+} (0-Ca^{2+}) and 1 μM nifedipine on spontaneous Ca^{2+} transients and contractions. Representative recoding (C) and statistical evaluation (D) showing the concentration-response curve obtained by cumulative addition of low concentrations of nifedipine. Data are expressed as relative percentage of the control (amplitude before treatment of nifedipine). Results are expressed as mean±SEM of six experiments. *Control vs Nifedipine ($p<0.05$).

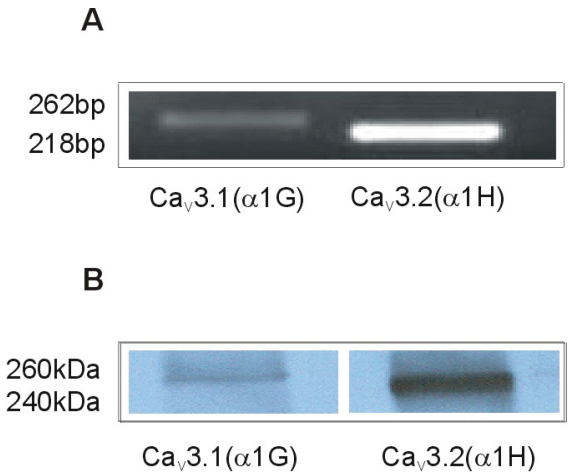

Fig. 2. Expression of mRNAs and proteins for α subunits (α1G and α1H) of T-type Ca^{2+} channel in longitudinal muscle layer of pregnant rat myometrium. Representative data of RT/PCR (A) and western blot (B). Immunoblots are representative of four independent preparations. The PCR was performed with 35 cycles and PCR products were followed by electrophoresis on a 1% agarose gel.

units were found to be expressed in longitudinal muscle layer of rat uterus.

Effect of T-type Ca^{2+} channel blockers on spontaneous Ca^{2+} transients and contractions

Effects of three different T-type Ca^{2+} channel blockers on spontaneous Ca^{2+} transients and contractions of uterus strips are shown in Fig. 3. Cumulative addition of mibefradil produced concentration-dependent inhibition of amplitude and frequency of spontaneous Ca^{2+} transients and contractions. The threshold concentration of mibefradil to produce an inhibitory effect on the amplitude and frequency for the Ca^{2+} transients and contractions was 0.5 μM. The mean IC_{50} values for mibefradil to inhibit the amplitude and frequency were 1.31×10^{-6} M and 7.97×10^{-7} M for Ca^{2+} transients, and 1.76×10^{-6} M and 7.97×10^{-7} M for contractions. NNC 55-0396 had similar inhibitory effect with mibefradil. The mean IC_{50} values for NNC 55-0396 to inhibit the amplitude and frequency were 4.31×10^{-6} M and 4.08×10^{-6} M for Ca^{2+} transients, and 5.24×10^{-6} M and 4.82×10^{-6} M for contractions. Cumulative addition of nickel produced a concentration-related inhibitory effect of the amplitude and frequency of spontaneous Ca^{2+} transients and contractions. The mean IC_{50} values for nickel inhibition of the amplitude and frequency were 1.94×10^{-4} M and

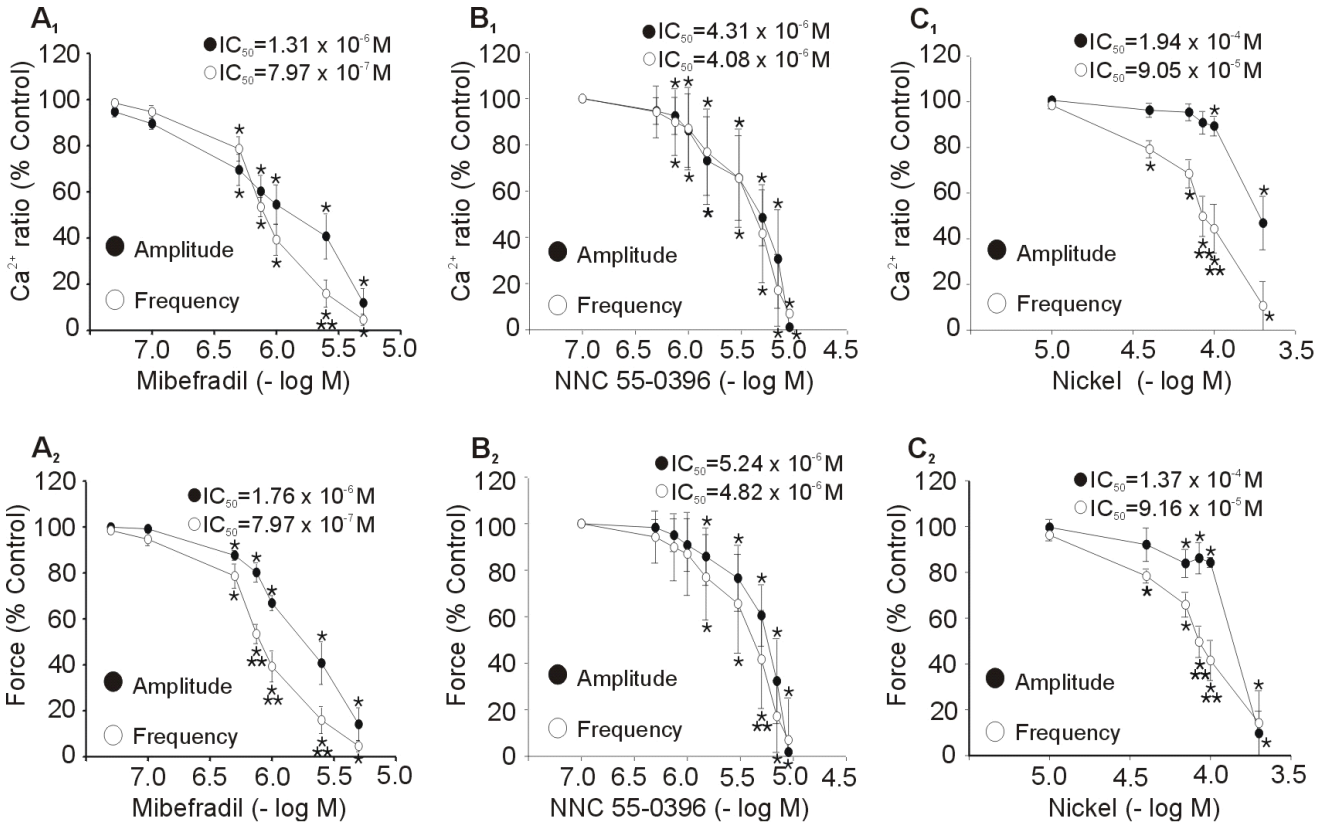

Fig. 3. Dose-response curve for the effect of T-type Ca^{2+} channel blockers on the spontaneous Ca^{2+} transients and contractions. (A_1, B_1, C_1) Concentration-related reduction of the amplitude and frequency of spontaneous Ca^{2+} transients. (A_2, B_2, C_2) concentration-related reduction of the amplitude and frequency of spontaneous contractions. Mibefradil (A), NNC 55-0396 (B), and nickel (C) were added cumulatively. Data are expressed as relative percentage of control (amplitude before treatment of blockers). Results are expressed as mean±SEM of seven experiments. *Control vs Blockers, **Amplitude vs Frequency ($p<0.05$).

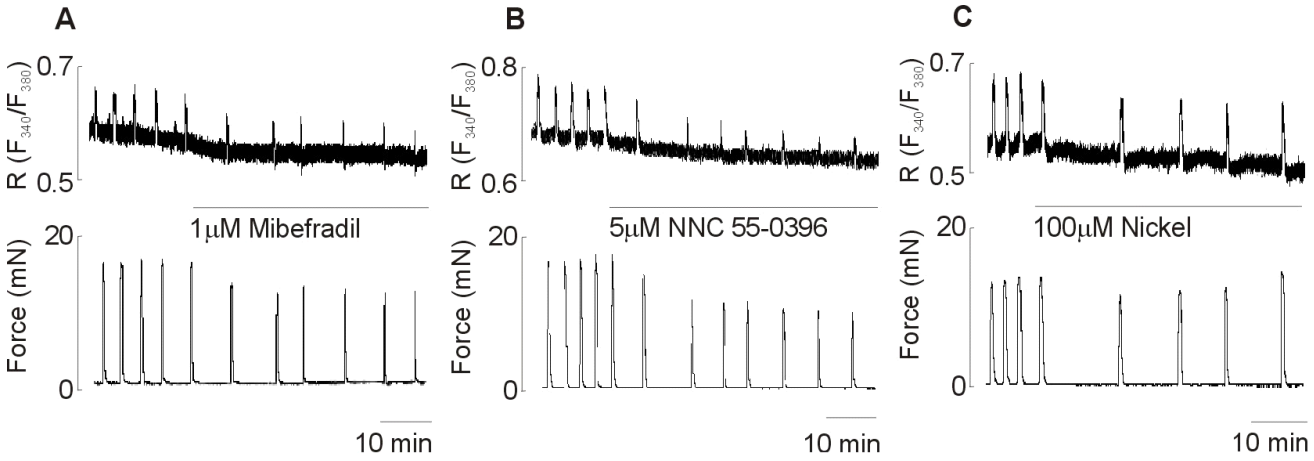

Fig. 4. Representative recording for the effect of T-type Ca^{2+} channel blockers on spontaneous Ca^{2+} transients and contractions. Spontaneous Ca^{2+} transients (top) and contractions (bottom) before and during 1 μM mibefradil (A), 5 μM NNC 55-0396 (B), and 100 μM nickel (C) application. Data are representative of ten independent preparations.

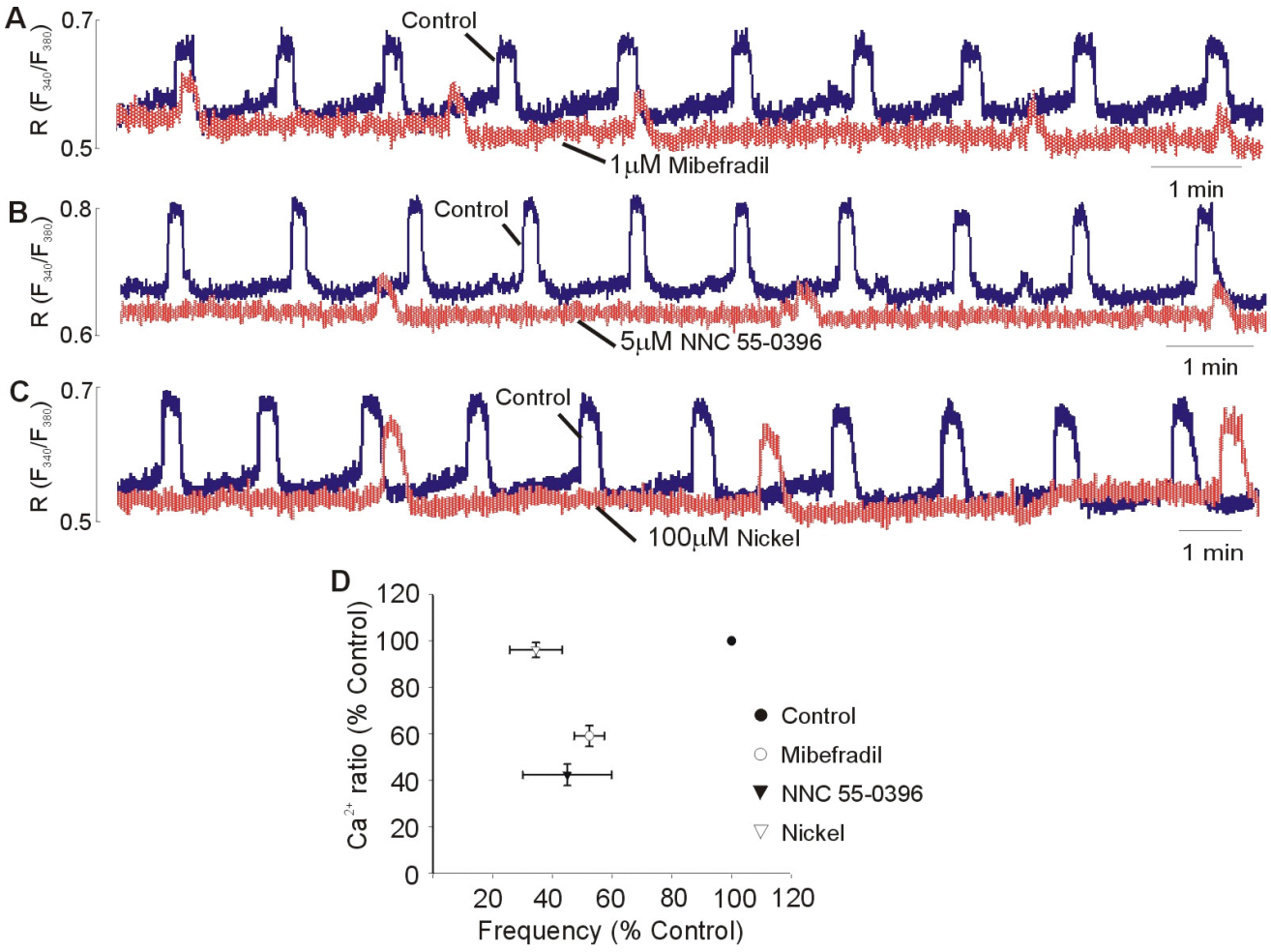

Fig. 5. Effect of the T-type Ca^{2+} channel blockers on the frequency and slope of rising phase of spontaneous Ca^{2+} transients. Superimposed spontaneous Ca^{2+} transients under control conditions and in presence of 1 μM Mibefradil (A), 5 μM NNC 55-0396 (B), and 100 μM nickel (C), respectively. Each blocker was added in the bath solution after spontaneous Ca^{2+} transients were stable. Statistical evaluation (D) showing the Ca^{2+} ratio and frequency obtained by adding mibefradil, NNC 55-0396, and nifedipine. Data are expressed as relative percentage of control. Results are expressed as mean±SEM of six experiments.

9.05×10^{-5} M for Ca^{2+} transients, and 1.37×10^{-4} M, 9.16×10^{-5} M for contractions. Mibefradil and NNC 55-0396 produced a similar concentration-response curve for inhibition of the amplitude and frequency, although the IC_{50} for frequency was lower than it for amplitude. However, nickel produced a steeper concentration-response curve for inhibition of the frequency than that of the amplitude.

To investigate the blockers-related reduction of the amplitude and frequency of spontaneous Ca^{2+} transients and contractions, effects of each IC_{50} of T-type Ca^{2+} channel blockers on spontaneous Ca^{2+} transients and contractions were tested. As shown in Fig. 4, 1 μM mibefradil (Fig. 4A) and 5 μM NNC 55-0396 (Fig. 4B) reduced the amplitude as well as frequency for the spontaneous Ca^{2+} transients and contractions, respectively. However, 100 μM nickel (Fig. 4C) reduced the frequency of spontaneous Ca^{2+} transients and contractions but not the amplitude of them.

Comparison of frequency and slope of rising phase of spontaneous Ca^{2+} transients in the presence and absence of T-type Ca^{2+} channel blockers

To clarify the role of the T-type Ca^{2+} channels on the frequency of the spontaneous Ca^{2+} transients and contractions, effects of the T-type Ca^{2+} channel blockers on the frequency and slope of the rising phase of Ca^{2+} transients were tested. Fig. 5 showed the spontaneous Ca^{2+} transients in the presence and absence of blockers. Mibefradil (Fig. 5A), NNC 55-0396 (Fig. 5B), and nickel (Fig. 5C) reduced the frequency of the spontaneous Ca^{2+} transients and attenuated the slope of rising phase of the spontaneous Ca^{2+} transient. Especially, nickel has more sensitive inhibitory effect on the inhibition of frequency compared than other inhibitors, mibefradil or NNC 55-0396 (Fig. 5D).

Effect of T-type Ca^{2+} channel blockers on the 70 mM KCl-induced contractions

From the above data it is clear that mibefradil and NNC 55-0396 reduce both the frequency and amplitude of the spontaneous Ca^{2+} transients and contractions. To determine whether T-type Ca^{2+} channel blockers used in the present study affect the L-type Ca^{2+} channels, effects of T-type Ca^{2+} channel blockers on the 70 mM KCl-induced contraction were examined. As shown in Fig. 6A, 1 μM nifedipine completely inhibited the 70 mM KCl-induced increase

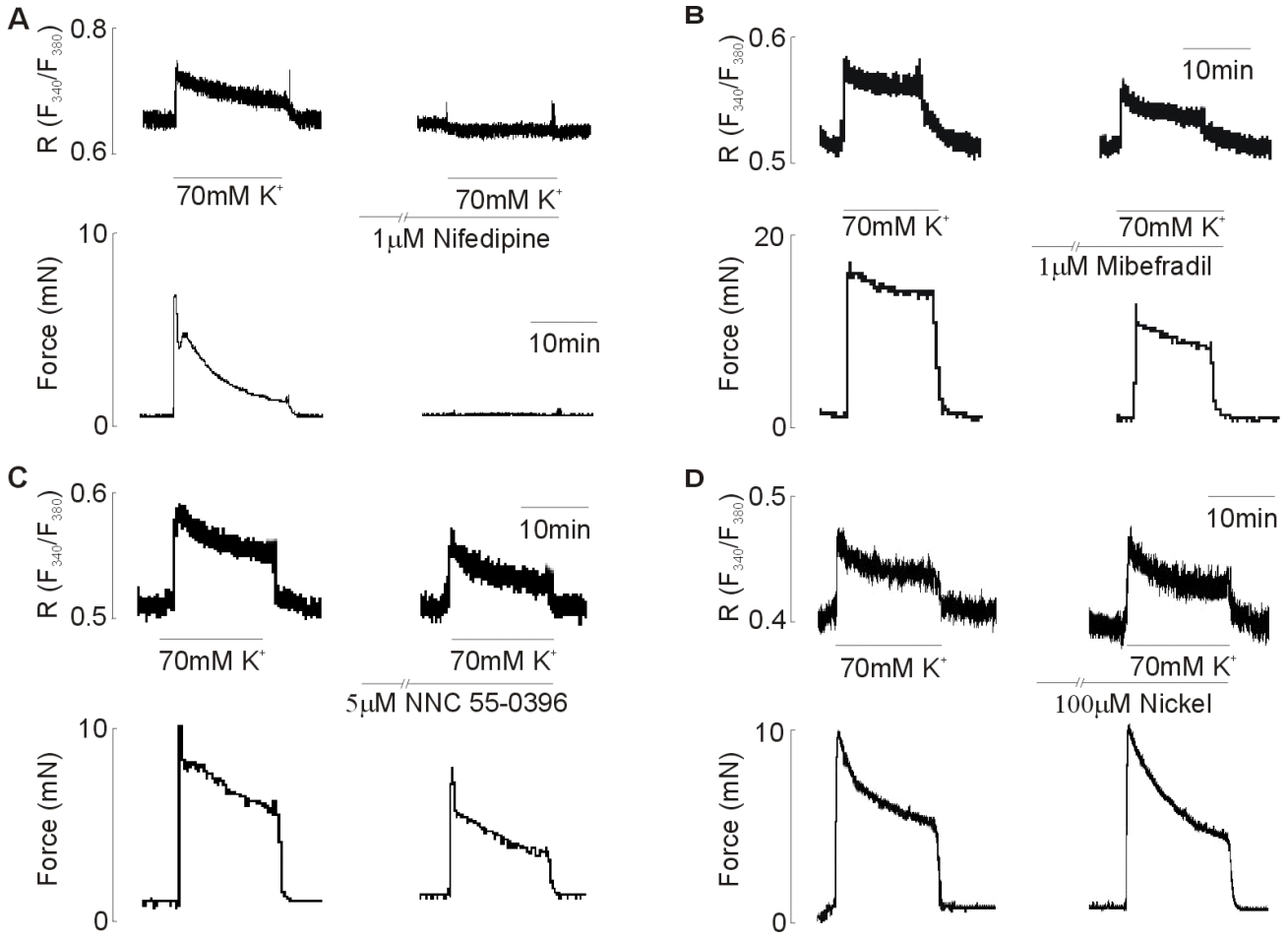

Fig. 6. Effect of (A) nifedipine (1 μM), (B) mibefradil (1 μM), (C) NNC 55-0396 (5 μM), and (D) nickel (100 μM) on the 70 mM KCl-induced increase in $[Ca^{2+}]_i$ and force. All drugs were added for 10 min before 70 mM KCl-induced contraction. Data are representative of seven~nine independent preparations.

in the $[Ca^{2+}]_i$ and contraction. 1 μM Mibefradil and 5 μM NNC55-0396 inhibited the 70 mM KCl-induced increase in the $[Ca^{2+}]_i$ and force, respectively. Mibefradil caused 47.48±5.53% ($p<0.05$) decrease in $[Ca^{2+}]_i$ and 16.15±6.54% decrease in force compared to that induced by 70 mM KCl (n=7, Fig. 6B). NNC 55-0396 also caused 22.56±2.08% ($p<0.05$) decrease in $[Ca^{2+}]_i$ and 13.18±6.54% decrease in force compared to that induced by 70 mM KCl (n=8, Fig. 6C). However, in contrast, there was little effect on $[Ca^{2+}]_i$ and force in response to 100 μM nickel. The decrease in $[Ca^{2+}]_i$ and force was 4.73±2.05% and 3.1±2.93% (n=9), respectively, of the rise in $[Ca^{2+}]_i$ and force produced by 70 mM KCl.

DISCUSSION

In this study, it has been shown that the T-type Ca^{2+} channels are expressed on the pregnant rat myometrium and the T-type Ca^{2+} channels play an important role in the generation of the spontaneous phasic Ca^{2+} transients and contractions. Furthermore, our data suggests that Ca^{2+} influx through T-type Ca^{2+} channels may regulate the frequency of spontaneous phasic contractions.

The generation of the spontaneous phasic contractions is due to the ability of a cell to fire a regenerative action potential. Thus, it is important to understand the mechanisms underlying the spontaneous depolarization between action potentials in the uterine smooth muscle. It has been known that L-type Ca^{2+} channel is the major source of Ca^{2+} influx for contraction in both human and rat (Mironneau, 1973; Ohya and Sperelakis, 1989; Young et al., 1993). It is consistent with our results that the spontaneous Ca^{2+} transients and contractions were abolished by the removal of external Ca^{2+} and treatment of 1 μM nifedipine, L-type Ca^{2+} channel blocker.

The membrane potential in uterine smooth muscle cells is not stable, and in some cells, termed pacemakers, a spontaneous depolarization of the membrane occurs. The exact nature of the membrane currents and channels leading this depolarization in the myometrium is not known (Parkington and Coleman, 1988; Coleman and Parkington, 1990; Wray et al., 2003). In the present study, although nifedipine completely abolished the spontaneous Ca^{2+} transients and contractions, L-type Ca^{2+} channels may be not involved in the generation of the slow depolarization. L-type Ca^{2+} channels have a high voltage activation threshold (around -40 mV) (Jmari et al., 1986; Honore et al., 1989). Parkington et al. (1999) have shown that the value of the resting membrane potential recorded in the myometrium ranged from -80 to -55 mV between species. Taken together, these previous results represent that L-type Ca^{2+} channel may be involved in the firing of action potentials, but not in the generation of slow depolarization. In the present study, we also determined the role of L-type Ca^{2+} channel on the spontaneous Ca^{2+} transients and contractions by treatment of low concentration of nifedipine, L-type Ca^{2+} channel blockers. Cumulative addition of low concentration of nifedipine (Fig. 1), which did not completely abolished spontaneous contractions, produced a decrease in the amplitude of spontaneous contractions. However, in contrast, the frequency of spontaneous contractions did not significantly changed by nifedipine. This means that there should be other types of Ca^{2+} channels, which may be involved in the slow membrane depolarization to aid in the opening of the L-type Ca^{2+} channel.

As a candidate, the T-type Ca^{2+} channel has a low activation threshold (around -60 mV) and a rapid inactivation (Perez-Reyes, 2003). In addition, a number of studies have been reported that the T-type Ca^{2+} channel is involved in the regulation of the frequency of action potential and the spontaneous contractions in various types of muscles such as the sinoarterial node of a rabbit heart (Doerr et al., 1989) and the detrusor smooth muscle of a guinea pig (Chow et al., 2003).

To determine the expression of the T-type Ca^{2+} channel in the pregnant rat myometrium, we examined the expression of the two T-type α subunits (α1G and α1H) by methods of RT/PCR and western blot. We observed that the mRNAs and proteins of α1G and α1H subunits are expressed in longitudinal strips of rat myometrium. These results are consistent with a previous study that both α1G and α1H are differentially expressed throughout gestation in the different layers of rat myomerium (Ohkubo et al., 2005).

To elucidate the role of the T-type Ca^{2+} channel in the spontaneous Ca^{2+} transients and contractions of rat myometrium, we observed the effect of the T-type Ca^{2+} channel blockers on the change of the spontaneous Ca^{2+} transients and contractions. In the present study, mibefradil, NNC 55-0396 and nickel were used as T-type Ca^{2+} channel blockers. Until recently, the lack of selective T-type Ca^{2+} channel blockers has hindered the attempts to investigate the role of T-type Ca^{2+} channels. Mibefradil has been known that a novel Ca^{2+} channel antagonist from the new chemical structural class of bensimidazolyl-substituted teraline derivatives (Billman and Hermsmeyer, 1994). In the vascular smooth muscle, a low concentration of mibefradil selectively blocked T-type Ca^{2+} channels (Mishra and Hermsmeyer, 1994). However, in contrast, the recent investigation reported that mibefradil also blocked the L-type Ca^{2+} channel by active metabolite produced via intracellular hydrolysis. Therefore, non-hydrolyzable analogue of mibefradil, NNC 55-0396, was developed as a selective blocker of the T-type Ca^{2+} channel (Huang et al., 2004). We showed that cumulative addition of mibefradil and NNC 55-0396 produced concentration-dependent inhibition of frequency as well as amplitude of spontaneous Ca^{2+} transients and contractions, respectively. These blockers also inhibited both the frequency and amplitude of Ca^{2+} transients and contractions at IC_{50} of these blockers. The results are consistent with a previous study that mibefradil inhibited the frequency as well as amplitude of uterine contractility (Asokan et al., 2002). These results suggested that mibefradil and NNC 55-0396 have an other side effect beside the inhibition of T-type Ca^{2+} channels. To evaluate whether mibefradil and NNC 55-0396 block L-type Ca^{2+} channels, we determined the effect of mibefradil and NNC 55-0396 on the high K^+-induced contractions. Mibefradil and NNC 55-0396 significantly inhibited the amplitude of high K^+-induced increase in $[Ca^{2+}]_i$ and force. According to the previous report, high K^+-induced contraction is due to Ca^{2+} influx through L-type Ca^{2+} channel by membrane depolarization (Shmigol et al., 1998; Coleman et al., 2000). In the present study, we also showed that 1 μM nifedipine, L-type Ca^{2+} channel blocker, completely inhibited high K^+-induced contraction. Therefore, these blockers not only block Ca^{2+} influx through T-type Ca^{2+} channels more selectively but also block it through L-type Ca^{2+} channel.

To further determine the role of T-type Ca^{2+} channels

on the spontaneous Ca^{2+} transients and contractions, we used nickel as a blocker of T-type Ca^{2+} channel. Nickel has been proposed as a selective blocker of the T-type Ca^{2+} channel depending on concentration (Lee et al., 1999). In the present study, cumulative addition of nickel produced a concentration-related inhibitory effect on frequency and amplitude of spontaneous Ca^{2+} transients and contractions, but the inhibition was more sensitive in frequency than in amplitude. In IC_{50} of 100 μM nickel produced an inhibition of frequency of the spontaneous Ca^{2+} transients and contractions. However, nickel has little effect on the amplitude of them. IC_{50} of nickel for the amplitude and frequency of spontaneous contractions was around 100 μM (91~137 μM). In some study, 100~200 μM nickel inhibits preferentially T-type Ca^{2+} channels (Tytgat et al., 1990; Sui et al., 2001). It is similar to the IC_{50} in oocytes (Lee et al., 1999). We also showed that 100 μM nickel had no effect on the high K^+-induced contractions. Therefore, the inhibitory effect of nickel to the frequency of spontaneous Ca^{2+} transients and contractions may be due to inhibition of Ca^{2+} influx through T-type Ca^{2+} channels.

Finally, to determine whether T-type Ca^{2+} channels are involved in the generation of spontaneous slow depolarization in rat myometrium, we compared the effect of three blockers on the slope of rising phase of spontaneous Ca^{2+} transients. All three different T-type Ca^{2+} channel blockers decreased the slope of the initial rising phase of Ca^{2+} transients and frequency. Although we did not measure the membrane potential for the change of rising phase of slow depolarization in the present study, the change of Ca^{2+} transients can represent the change of membrane potentials. Furthermore, the t-type window current (the balance between the voltage-dependence of activation and inactivation) may be around about the resting membrane potential of myometrial cells and therefore able theoretically to contribute to action potential firing (Taggart and Tribe, 2007). Therefore, T-type Ca^{2+} channels may be involved in the generation of spontaneous Ca^{2+} transients and the modulation of the frequency of spontaneous Ca^{2+} transients.

ACKNOWLEDGEMENTS

This study was supported by a faculty research grant of Yonsei University of Medicine for 2007 (No. 6-2007-0180).

REFERENCES

Asokan KT, Sarkar SN, Mishra SK, Raviprakash V. Effects of mibefradil on uterine contractility. *Eur J Pharmacol* 455: 65−71, 2002.

Billman GE. Ro 40-5967, a novel calcium channel antagonist, protects against ventricular fibrillation. *Eur J Pharmacol* 229: 179−187, 1992.

Blanks AM, Zhao ZH, Shmygol A, Bru-Mercier G, Astle S, Thornton S. Characterization of the molecular and electrophysiological properties of the T-type calcium channel in human myometrium. *J Physiol* 581(Pt 3): 915−926, 2007.

Challis JRG, Matthews SG, Gibb W, Lye SJ. Endocrine and paracrine regulation of birth at term and preterm. *Endocrine Rev* 21: 514−550, 2000.

Chien EK, Saunders T, Phillippe M. The mechanisms underlying Bay K 8644-stimulated phasic myometrial contractions. *J Soc Gynecol Invest* 3: 106−112, 1996.

Chow KY, Wu C, Sui GP, Fry CH. Role of the T-type Ca^{2+} current on the contractile performance of guinea pig detrusor smooth muscle. *Neurourol Urodyn* 22: 77−82, 2003.

Coleman HA, Hart JDE, Tonta MA, Parkington HC. Changes in the mechanisms involved in uterine contractions during pregnancy in guinea-pigs. *J Physiol* 523: 785−798, 2000.

Coleman HA, Parkington HC. The role of membrane potential in the control of uterine motility. In: Carsten ME, Miller JD ed, *Uterine function: Molecular and cellular aspects*. New York: Plenum Press, p 195−248, 1990.

Collins PL, Moore JJ, Idriss E, Kulp TM. Human fetal membranes inhibit calcium L-channel activated uterine contractions. *Am J Obstet Gynecol* 175: 1173−1179, 1996.

Doerr T, Denger R, Trautwein W. Calcium currents in single SA nodal cells of the rabbit heart studied with action potential clamp. *Pflugers Arch* 413: 599−603, 1989.

Honore E, Amedee T, Martin C, Dacquet C, Mironneau C, Mironneau J. Calcium channel current and its sensitivity to (+) isradipine in cultured pregnant rat myometrial cells. *Pflugers Arch* 414: 477−483, 1989.

Huang L, Keyser BM, Tagmose TM, Hansen JB, Taylor JT, Zhuang H, Zhang M, Ragsdale DS, Li M. NNC 55-0396[(1S,2S)-2-(2-(N-[(3-benzimidazol-2-yl)propyl]-*N*-methylamino)ethyl)-6-fluoro-1,2,3,4-tetrahydro-1-isopropyl-2-naphytyl cyclopropanecarboxylate dihydrochloride]: a new selective inhibitor of T-type calcium channels. *J Pharmacol Exp Therap* 309: 193−199, 2004.

Inoue Y, Sperelakis N. Gestational change in Na^+ and Ca^{2+} current densities in rat myometrial smooth muscle cells. *Am J Physiol* 260: C658−C663, 1991.

Jmari K, Mironneau C, Mironneau J. Inactivation of calcium channels current in rat uterine smooth muscle: evidence for calcium and voltage-mediated mechanisms. *J Physiol* 380: 111−126, 1986.

Knock GA, Aaronson PI. Calcium antagonistic properties of the cyclooxygenase-2 inhibitor nimesulide in human myometrial myocytes. *Br J Pharmacol* 127: 1470−1478, 1999.

Lee JH, Gomora JC, Cribbs LL, Perez-Reyes E. Nickel block of three cloned T-type calcium channels: low concentrations selectively block α1H. *Biophys J* 77: 3034−3042, 1999.

Mershon JL, Mikala G, Schwartz A. Changes in the expression of the L-type voltage-dependent calcium channel during pregnancy and parturition in the rat. *Biol Reprod* 51: 993−999, 1994.

Mironneau J. Excitation-contraction coupling in voltage clamped uterine smooth muscle. *J Physiol* 233: 127−141, 1973.

Mishra SK, Hermsmeyer K. Selective inhibition of T-type Ca^{2+} channels by Ro 40-5967. *Circ Res* 75: 144−148, 1994.

Ohkubo T, Kawarabayashi T, Inoue Y, Kitamura K. Differential expression of L-and T-type calcium channels between longitudinal and circular muscles of the rat myometrium during pregnancy. *Gynecol Obstet Invest* 59: 80−85, 2005.

Ohya Y, Sperelakis N. Fast Na^+ and slow Ca^{2+} channels in single uterine muscle cells from pregnant rats. Am *J Physiol* 257: C408−C412, 1989.

Parkington HC, Coleman HA. Ionic mechanisms underlying action potentials in myometrium. *Clin Exp Pharmacol Physiol* 15: 657−665, 1988.

Parkington HC, Coleman HA. Excitability in uterine smooth muscle. *Front Horm Res* 27: 179−200, 2001.

Parkington HC, Tonta MA, Brennecke SP, Coleman HA. Contractile activity, membrane potential, and cytoplasmic calcium in human uterine smooth muscle in the third trimester of pregnancy and during labor. *Am J Obstet Gynecol* 181: 1145−1151, 1999.

Perez-Reyes E. Molecular physiology of low-voltage-activated T-type calcium channels. *Physiol Rev* 83: 117−161, 2003.

Riemer RK, Heymann MA. Regulation of uterine smooth muscle function during gestation. *Pediatrics Res* 44: 615−627, 1998.

Shmigol AV, Eisner DA, Wray S. Properties of voltage-activated $[Ca^{2+}]_i$ transients in single smooth muscle cells isolated from pregnant rat uterus. *J Physiol* 511: 803−811, 1998.

Sui GP, Wu C, Fry CH. Inward calcium currents in cultured and freshly isolated detrusor muscle cells: evidence of a T-type calcium current. *J Urol* 165: 621−626, 2001.

Taggart MJ, Tribe RM. Cellular ionic mechanisms controlling

uterine smooth muscle contraction: effects of gestational state. In: Savineau JP ed, *New Frontiers in Smooth Muscle Biology and Physiology*. India: Research Signpost, p 523−549, 2007.

Tytgat J, Vereecke J, Carmeliet E. Combined study of sodium current and T-type calcium current in isolated cardiac cells. *Pflugers Arch* 417: 142−148, 1990.

Wray S, Jones K, Kupittayanant S, Li Y, Matthew A, Monir-Bishty E, Noble K, Pierce SJ, Quenby S, Shmygol AV. Calcium signaling and uterine contractility. *J Soc Gynecol Invest* 10: 252−264, 2003.

Yeon DS, Kim JS, Ahn DS, Kwon SC, Kang BS, Morgan KG, Lee YH. Role of protein kinase C- or RhoA-induced Ca^{2+} sensitization in stretch-induced myogenic tone. *Cardiovas Res* 53: 431−438, 2002.

Young RC, Smith LH, McLaren MD. T-type and L-type calcium currents in freshly dispersed human uterine smooth muscle cells. *Am J Obstet Gynecol* 169: 785−792, 2003.