Role of T-type Ca^{2+} channels in the spontaneous phasic contraction of pregnant rat uterine smooth muscle

Si-Eun Lee

Department of Medical Science,
the Graduate School, Yonsei University

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Directed by Professor Young-Ho Lee

The Master's Thesis
submitted to the Department of Medical Science,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree of
Master of Medical Science

Si-Eun Lee

June 2008

This certifies that the Master's
Thesis of Si-Eun Lee is approved.

Thesis Supervisor : Young-Ho Lee

Thesis Committee Member : Duck-Sun Ahn

Thesis Committee Member : Sang -Wook Bai

The Graduate School
Yonsei University

June 2008

Acknowledgements

While I had been working on my thesis for two years, I thought that it was like a long journey. Until I accomplished my thesis, my professor, Young-Ho Lee, was always there to teach and help me to move ahead in next step. And he waited with patient and encouraged me whenever I had difficulty in working. I would like to express my heartfelt gratitude to my professor. I also would like to express thanks to committee members, Duck-Sun Ahn and Sang-Wook Bai who gave me unstinted comments.

I especially thank to my coworkers, Young-Eun Cho, Soo-Kyoung Choi and Young-Hwan Kim, for helping me and inspiring me to complete my master's thesis. And I am very thankful to Shin-Woo Kang for helping me to prepare the experiment every day. I also thank to all members of department of physiology.

Last, I would like to my thanks to my family for endless love, belief, and unconditional support.

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Abstract

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It has been suggested that the 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. Two types of Ca^{2+} channel, L-type and T-type Ca^{2+} channel, have been described in the myometrium. Although there are many reports concerned with roles of L-type Ca^{2+} channels, the role of the T-type Ca^{2+} channels on the spontaneous phasic contractions has yet to be fully identified. Furthermore, the existence of T-type Ca^{2+} channels is not clear in the rat myometrium. The aim of this study was to investigate whether the T-type Ca^{2+} channels are expressed on the pregnant rat uterine smooth muscle and 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=22days). The expression of T-type Ca^{2+} channel mRNAs or protein levels was measured.

The uterus strips from pregnant rats exhibited spontaneous Ca^{2+} transients and contractions with a mean amplitude of 12.16±2.30 mN and mean frequency of

0.69±1.14 contractions/min (n=10). The spontaneous phasic contractions stopped and $[Ca^{2+}]_i$ fell upon changing to 0-Ca^{2+} solution or adding 1 μM nifedipine. 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, NNC55-0396, and nickel induced a concentration-dependent inhibition of the amplitude and frequency of the spontaneous Ca^{2+} transients and contractions. Mibefradil and NNC55-0396 produced a similar concentration-response curve for inhibition of the amplitude and frequency. However, nickel induced steeper concentration-response curve for inhibition of the frequency than that of the amplitude. In IC_{50} of these blockers, 1 μM mibefradil and 5 μM NNC 55-0396 reduced both the amplitude and the frequency of the spontaneous Ca^{2+} transients and contractions. However, 100 μM nickel reduced the frequency of the spontaneous Ca^{2+} transients and contractions, but not the amplitude of them. Mibefradil, NNC 55-0396, and nickel also attenuated the slope of rising phase of spontaneous Ca^{2+} transients consistent with the reduction of the frequency. Mibefradil and NNC 55-0396 inhibited the 70 mM KCl-induced increase in $[Ca^{2+}]_i$ and force, but neither the $[Ca^{2+}]_i$ nor force was inhibited by the treatment of nickel. Cumulative addition of low concentrations of nifedipine produced a decrease in the amplitude of the spontaneous Ca^{2+} transients and contractions. However, in contrast, the frequency of the spontaneous Ca^{2+} transients and contractions was not significantly changed by low concentration of nifedipine.

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. Furthermore, Ca^{2+} influx through T-type Ca^{2+} channels is concerned with main currents in the slow depolarization for spontaneous phasic contractions.

Key words: spontaneous phasic contraction, Ca^{2+} channels, mibefradil, NNC 55-0396, nickel, uterine smooth muscle

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Ⅰ. 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.[1, 2, 3]

It is well known that spontaneous phasic contraction of 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.[4] 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,[5] pharmacologic,[6, 7] and molecular studies.[8] 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 mucle.[1,2] 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[9] but not in rat myometrium.[10, 11] It has been also demonstrated that the mRNAs of T-type Ca^{2+} channel are expressed in human myometrium.[12] 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, dependent on gestational age and layer. [13]

As the T-type Ca^{2+} channel is thought to generate the pacemaker potential and to depolarize the plasma membrane allowing 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.[12] This 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.

Ⅱ. MATERIALS AND METHODS

1. 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 cold oxygenated normal Tyrode solution of following composed (mM): Glucose 12, NaCl 135, KCl 5.4, $MgCl_2$ 1.2, HEPES 10, $CaCl_2$ 2.5, pH7.4. Blood, placental tissue, and circular layer were gently removed and the longitudinal 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.

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℃ 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.

2. Reverse Transcription – Polymerase Chain Reaction

The longitudinal muscle layer was collected separately from endometrium, submucous tissues, and circular muscle layer in ice-cold normal Tyrode solution bubbled with 100 % O_2.

For the isolation of total RNA, the collected tissue was broken down using a pestle in 1 ml easy-BLUE^{TM} (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 d(T), 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^{TM} 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 mixures 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.

3. Western blot

Longitudinal strips were dissected and quick-frozen in dry ice and homogenized in

buffer containing Triton X100 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 ponceaus 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 (1:5000; 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.

4. Drugs and chemicals

The Following drugs were used: nifedipine (Sigma, St Louis, MO, USA), NNC55-0396 ([(1*S*,2*S*)-2-(2-(*N*-[(3-Benzimidazol-2yl)popyl]-*N*-methylamino)ethyl)-6-fluoro-1,2,3,4-tetrahyd-ro-1-isopropyl-2-naphtyl cyclopropanecarboxylate dihyrochlo-ride]) (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.

5. Statistics

Data are expressed as mean ± S.E. 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 Student's *t*-test. Significant differences were taken at the $p < 0.05$ level.

Ⅲ. RESULTS

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

The uterus strips from pregnant rats exhibited spontaneous rhythmic Ca^{2+} transients and contractions in normal Tyrode solution, with a mean 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 VDCC, 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.

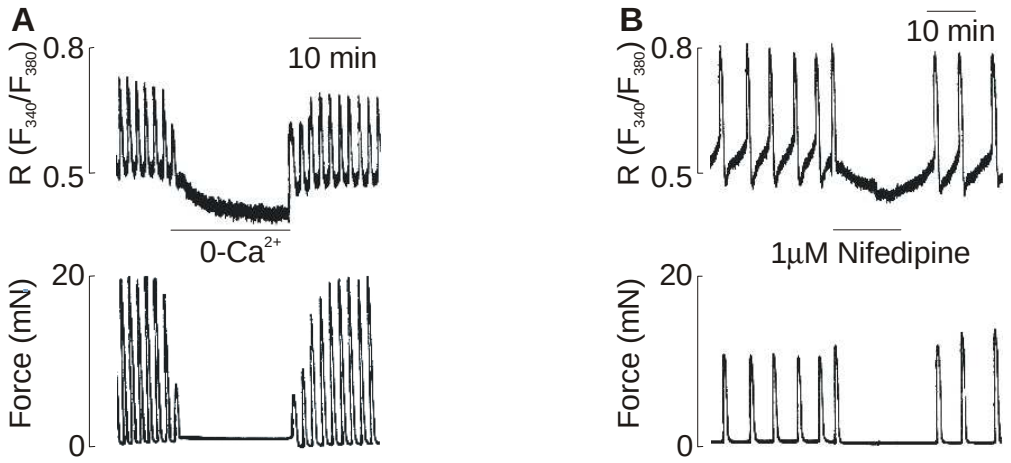

Fig. 1. Effect of removing external Ca^{2+} and nifedipine on spontaneous Ca^{2+} transients (top) and contractions (bottom). A. Effect of removing external Ca^{2+} ($0\text{-}Ca^{2+}$) on spontaneous Ca^{2+} transients and contractions. B. Effect of 1 μM nifedipine on spontaneous Ca^{2+} transients and contractions.

2. 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 L-type Ca^{2+} channel blocker, was tested. As shown in Fig. 2, 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.

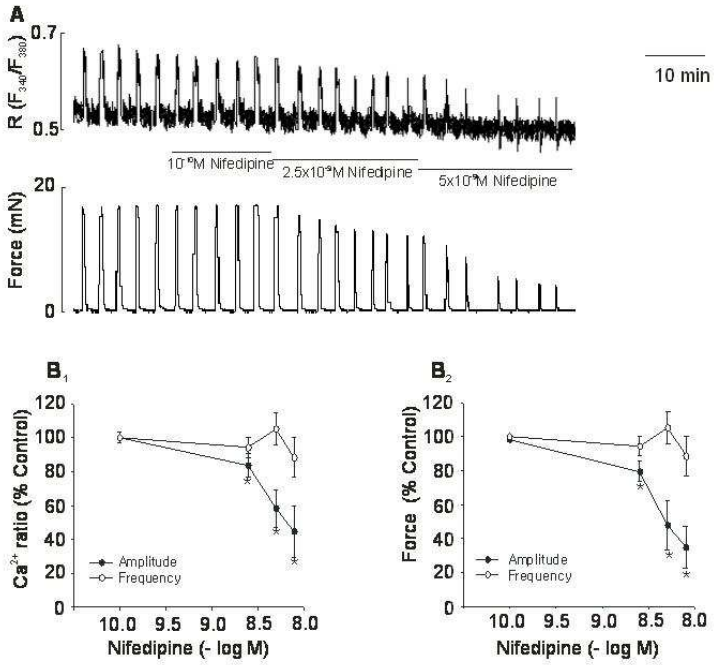

Fig. 2. Effect of low concentration of nifedipine on spontaneous Ca^{2+} transients and contractions. Representative recoding (A) and statistical evaluation (B) showing the concentration-response curve obtained by cumulative addition of nifedipine. Data is expressed as relative percentage of control. Results are expressed as mean ± S.E. *: Control vs Nifedipine (p<0.05)

3. 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. 3, two subunits were found to be expressed in longitudinal muscle layer of rat uterus.

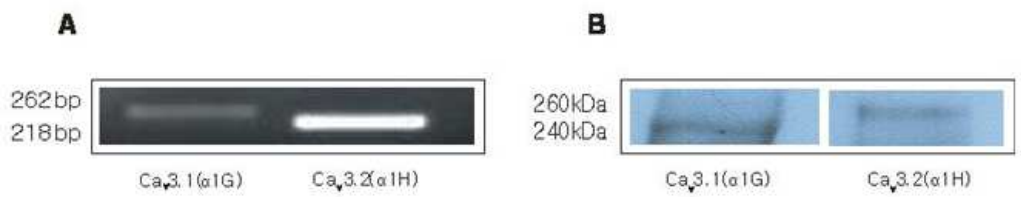

Fig. 3. Expression of mRNAs and proteins for α subunits (α1G and α1H) of T-type Ca^{2+} channel in longitudinal muscle layer of pregnant rat myometrium. Representative data of RT/PCR (A) and western blot (B). The PCR was performed with 35 cycles and PCR products were followed by electrophoresis on a 1 % agarose gel.

4. 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. 4. 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 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.

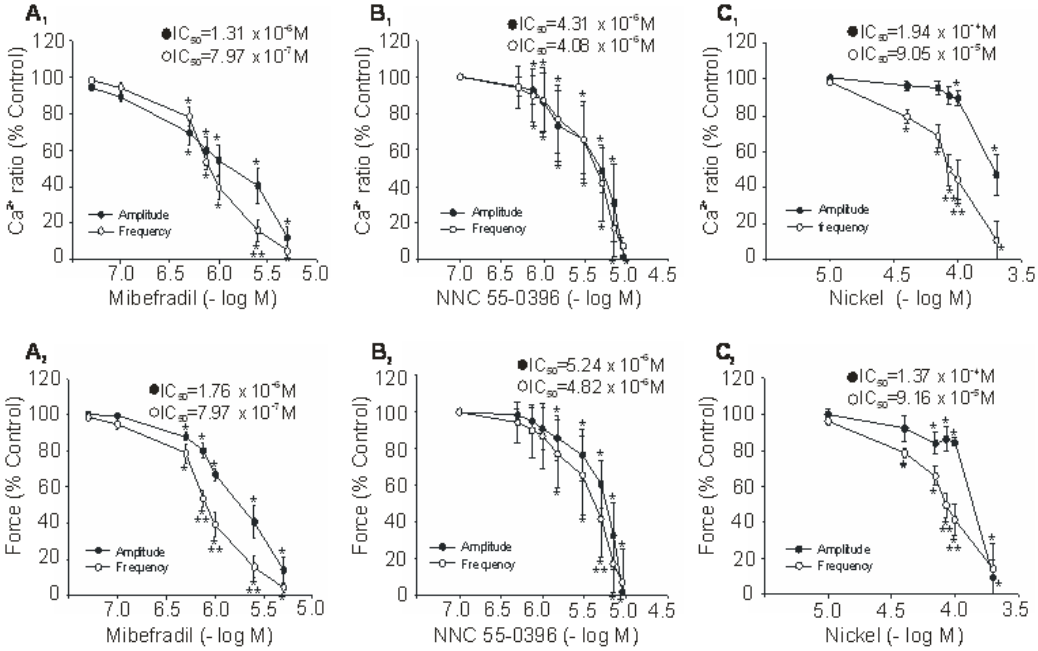

Fig. 4. Effect of T-type Ca^{2+} channel blockers on the spontaneous Ca^{2+} transients and contractions. A_1, B_1, C_1: Concentration-related reduction of the amplitude and frequency of spontaneous Ca^{2+} transients. A_2, B_2, C_2: concentration-related reduction of the amplitude and frequency of spontaneous contractions. Mibefradil (A), NNC 55-0396 (B), and nickel (C) were added cumulatively. Data is expressed as relative percentage of control. Results are expressed as mean ± S.E. *: Control vs Blockers, **: Amplitude vs Frequency (p<0.05)

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. 5, 1 μM mibefradil and 5 μM NNC 55-0396 reduced the amplitude as well as frequency for the spontaneous Ca^{2+} transients and contractions, respectively. However, 100 μM nickel reduced the frequency of spontaneous Ca^{2+} transients and contractions but not the amplitude of them.

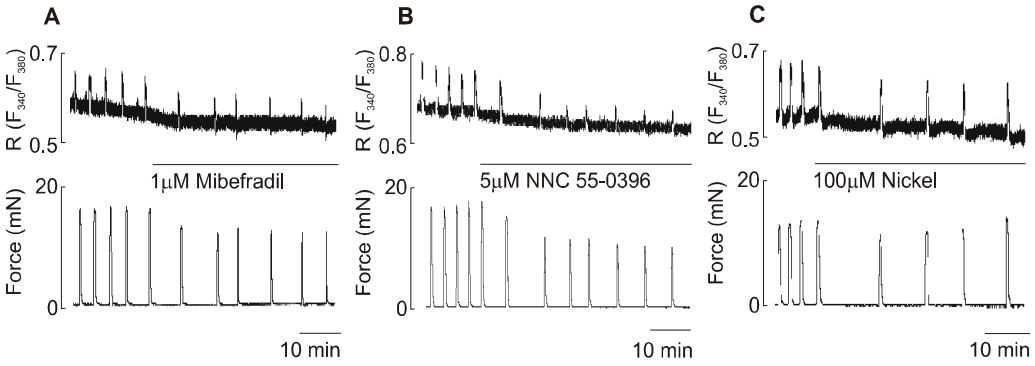

Fig. 5. Effect of T-type Ca^{2+} channel blockers on spontaneous Ca^{2+} transients and contractions. Spontaneous Ca^{2+} transients (top) and contractions (bottom) before and during 1 μM mibefradil (A), 5 μM NNC 55-0396 (B), and 100 μM nickel (C) application.

5. 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. 6 showed the spontaneous Ca^{2+} transients in the presence and absence of blockers. Mibefradil, NNC 55-0396, and nickel reduced the frequency of the spontaneous Ca^{2+} transients and attenuated the slope of rising phase of the spontaneous Ca^{2+} transient.

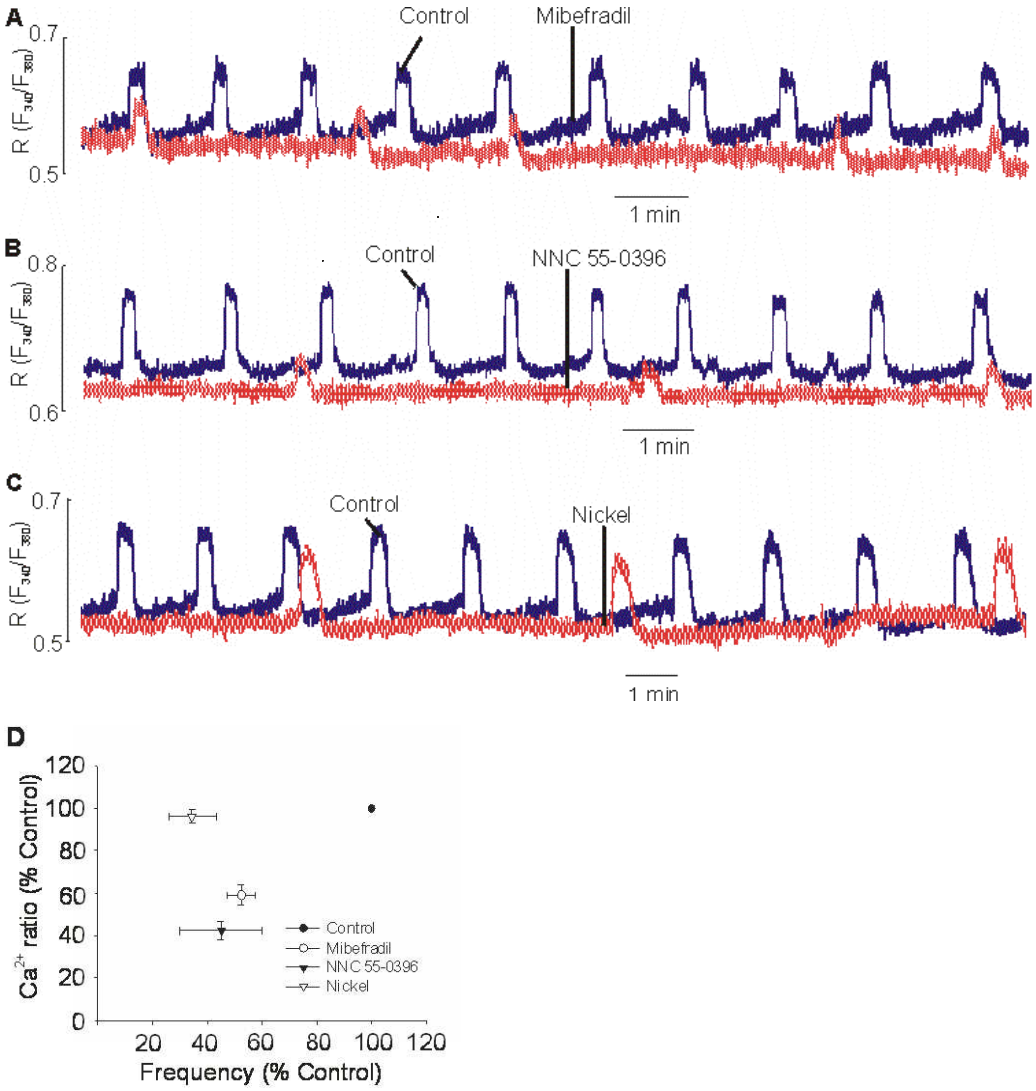

Fig. 6. Effect of the T-type Ca^{2+} channel blockers on the frequency and slope of rising phase of spontaneous Ca^{2+} transients. Superimposed spontaneous Ca^{2+} transients under control conditions and in presence of 1 μM Mibefradil (A), 5 μM NNC 55-0396 (B), and 100 μM nickel (C), respectively. Each 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 is expressed as relative percentage of control. Results are expressed as mean ± S.E.

6. 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. 7A, 1 μM nifedipine completely inhibited the 70 mM KCl-induced increase 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. 7B). 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. 7C). 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.

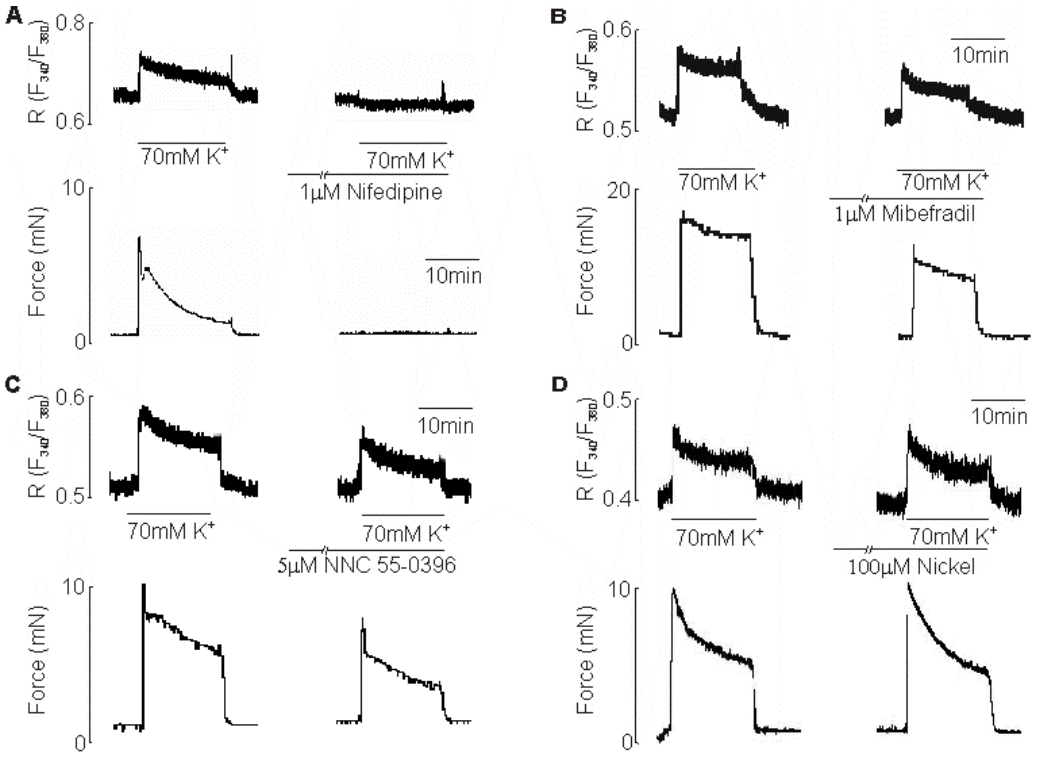

Fig. 7. Effect of nifedipine (1 μM), mibefradil (1 μM), NNC 55-0396 (5 μM), and nickel (100 μM) on the 70 mM KCl-induced increase in $[Ca^{2+}]_i$ and force. All drugs were added for 10 min before 70 mM KCl-induced contraction.

Ⅳ. 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, we have shown that Ca^{2+} influx through T-type Ca^{2+} channels is concerned with main currents in the slow depolarization for 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.[9, 11, 14] 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.[4, 5, 15] 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).[16, 17] Parkington and Coleman have shown that the value of the resting membrane potential recorded in the myometrium ranged from -80 to -55 mV between species.[18] 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. 2) 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.[19] 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[20] and the detrusor smooth muscle of a guinea pig.[21]

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.[13]

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.[22] In the vascular smooth muscle, a low concentration of mibefradil selectively blocked T-type Ca^{2+} channels.[23] 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.[24] 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.[25] These results suggested that mibefradil and NNC 55-0396 have an other side effect. 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.[26, 27] In the present study, we also showed that nifedipine, L-type Ca^{2+} channel blocker, completely inhibited spontaneous Ca^{2+} transients and contractions. 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.[28] 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.[29, 30] It is similar to the IC_{50} in oocytes.[31] 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. 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.

In summary, this study shows 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. Furthermore, Ca^{2+} influx through the T-type Ca^{2+}

channels concerned with main currents in the slow depolarization for spontaneous phasic contractions.

Ⅴ. CONCLUSION

The present study investigated the roles of T-type Ca^{2+} channels on the spontaneous Ca^{2+} transients and contractions of the pregnant rat myometrium by measuring the contractile responses in the longitudinal strip, the changes in $[Ca^{2+}]_i$, and the expression of the mRNAs and proteins of T-type Ca^{2+} channel α subunits. These results are as follows.

The uterus strips from pregnant rats exhibited spontaneous Ca^{2+} transients and contractions with a mean amplitude of 12.16±2.30 mN and mean of frequency of 0.69±1.14 contractions/min (*n*=10). The spontaneous phasic contractions stopped and $[Ca^{2+}]_i$ fell upon changing to 0-Ca^{2+} solution or adding 1 μM nifedipine. 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, and nickel induced concentration-dependent inhibition of the amplitude and frequency of the spontaneous Ca^{2+} transients and contractions. Mibefradil and NNC 55-0396 produced a similar concentration-response curve for inhibition of the amplitude and frequency. However, nickel induced steeper concentration-response curve for inhibition of the frequency than that of the amplitude. In IC_{50} of these blockers, 1 μM mibefradil and 5 μM NNC 55-0396 reduced both the amplitude and the frequency of the spontaneous Ca^{2+} transients and contractions.

However, 100 μM nickel reduced the frequency of the spontaneous Ca^{2+} transients and contractions but not the amplitude of them. Mibefradil, NNC 55-0396, and nickel also attenuated the slope of rising phase of spontaneous Ca^{2+} transients consistent with the reduction of the frequency. Mibefradil and NNC 55-0396 inhibited the 70 mM KCl-induced increase in $[Ca^{2+}]_i$ and force, but neither the $[Ca^{2+}]_i$ nor force was inhibited by the treatment of nickel. Cumulative addition of low concentrations of nifedipine produced a decrease in the amplitude of the spontaneous Ca^{2+} transients and contractions. However, in contrast, the frequency of the spontaneous Ca^{2+} transients and contractions was not significantly changed by nifedipine.

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. Furthermore, Ca^{2+} influx through the T-type Ca^{2+} channels is concerned with main currents in the slow depolarization for spontaneous phasic contractions.

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Abstract (in Korean)

임신말기 쥐의 자궁평활근에서 유발되는 자발적 수축에서 T-type Ca^{2+} channel의 역할

<지도교수 이 영 호>

연세대학교 대학원 의과학과

이 시 은

임신 쥐의 자궁평활근에서 막전압 의존성 칼슘통로(Voltage-dependent Ca^{2+} channel)를 통한 칼슘 유입으로 인한 세포 내 칼슘농도의 증가는 자발적 수축에 중요한 역할을 하는 것으로 알려져 있다. 막전압 의존성 통로로서 T-type Ca^{2+} channel과 L-type Ca^{2+} channel이 자궁평활근에 존재한다고 알려져 있다. L-type Ca^{2+} channel의 경우, 그 역할에 대해 많은 연구가 되어있지만, T-type Ca^{2+} channel의 역할에 대한 연구는 아직 미미하며 특히 임신 쥐의 자궁 평활근에서 T-type Ca^{2+} channel의 존재의 여부도 불분명한 상태이다. 따라서 본 연구에서는 임신 쥐의 자궁평활근에 T-type Ca^{2+} channel이 존재하는지 조사해보고, 자발적 수축의 형성과 유지에 T-type Ca^{2+} channel을 통한 Ca^{2+}유입의 역할에 대해서 알아보고자 하였다. 임신 18-20일째의 쥐에서 자궁평활근을 적출하여 longitudinal strip으로 분리 후 장력과 세포 내 칼슘농도 변화를 동시에 측정하였고, RT-PCR과 western blot을 통해 T-type Ca^{2+} channel의 subunit들의 발현을 알아보았다.

임신 쥐로부터 얻은 자궁 평활근 절편에서 형성되는 자발적인 세포 내 칼슘 농도변화와 수축의 평균 크기는 12.16±2.30 mN였고, contractions/min (n=10)의 빈도를 보였다. 자발적 수축과 세포 내 칼슘 변화는 외부 Ca^{2+}의 제거와 막전압 의존성 통로 차단제인 nifedipine에 의해서 완전히 억제되는 것을 관찰하였다. RT-PCR과 western blot을 통해

T-type Ca^{2+} channel의 subunit인 α1G와 α1H의 발현을 확인할 수 있었다. 또한 자발적 수축에서 세포 내 칼슘농도 변화와 수축은 T-type Ca^{2+} channel 차단제인 mibefradil과 NNC 55-0396 그리고 nickel에 의해 농도 의존적으로 감소되는 것을 관찰하였는데 특히 Mibefradil과 NNC 55-0396을 농도 별로 처리한 결과, 비슷한 농도반응 곡선을 보였으나 nickel의 경우 수축의 크기에서 보다 빈도를 더욱 유의 하게 감소시키는 것을 관찰할 수 있었다. 농도반응 곡선을 통하여 얻은 IC_{50} 농도인 1 μM mibefradil과 5 μM NNC 55-0396의 T-type Ca^{2+} channel 차단제 처리에 의해 자발적인 세포 내 칼슘농도 변화와 수축에서 크기와 빈도가 모두 감소되는 것을 관찰하였다. 그러나 100 μM nickel 처리시 수축의 빈도만 유의한 감소를 보일 뿐 그 크기에는 거의 영향을 미치지 않았다. 이러한 세가지 T-type Ca^{2+} channel 차단제 모두 자발적 수축에서 Ca^{2+}농도의 slow rising phase의 기울기를 감소시키는 것을 확인하였다. Mibefradil과 NNC 55-0396이 L-type Ca^{2+} channel에 영향을 미치는지 알아보기 위해, mibefradil과 NNC 55-0396를 전처리 한 실험에서 70 mM KCl에 의한 수축과 세포 내 Ca^{2+}농도의 증가가 감소 되었지만 nickel은 70 mM KCl에 의한 수축과 세포 내 Ca^{2+}농도의 증가에 영향을 미치지 않았다. 낮은 농도의 nifedipine을 농도 별로 처리한 결과 자발적인 칼슘농도 변화와 수축의 크기는 감소되었으나 빈도에는 거의 영향을 미치지 않았다.

이상의 실험을 종합해 볼 때 임신 쥐의 자궁 평활근에 T-type Ca^{2+} channel이 발현되어 있으며 T-type Ca^{2+} channel은 자발적 칼슘농도의 변화와 수축에서 빈도를 조절하는데 중요한 역할을 할 것으로 생각된다.

핵심되는 말: 자발적 수축, 칼슘통로, mibefradil, NNC 55-0396, nickel, 자궁평활근