Ion channels in uterine smooth muscle cells to regulate contractions during term and preterm delivery: - Role of non-selective cation channels and gap junctions.

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Abstract

Uterine contractions are generated by calcium (Ca^{2+}) influx into myometrial cells which is regulated by the membrane potential of myometrial cells. Non-selective cation channels (NSCCs) associated with membrane potential were studied in rat myometrial cells using the patch-clamp method and molecular biological techniques. Two types of NSCC current in myometrial cells were detected. One is lanthanum (La) sensitive NSCC current and the other is adenosine triphosphate (ATP) induced current. Purinergic P2X7 receptor was determined to be the functional channel for ATP induced currents. These NSCCs are suggested to be mechanisms to generate spontaneous depolarizations, leading to rhythmical uterine contractions. Blocking of these NSCCs by magnesium ions (Mg^{2+}) may be one of the mechanisms for inhibiting uterine contractions in tocolysis. Cell-to-cell couplings through gap junction (GJ) channels increase at the end of pregnancy. We used the double patch-clamp technique on myometrial paired cells and characterized GJ currents. Most properties were comparable to those of connexin 43, indicating that connexin 43 is the main component of myometrial GJ. Junctional conductance between myometrial cells was enhanced sixfold in preterm delivering compared with preterm non-delivering rats. This enhancement is caused by an increase in the number of GJ channels. Finally, the expression of P2X receptors was examined in the myometrium, and P2X4 and P2X7 were expressed most strongly. The expression levels of these receptors increases in the late stages of pregnancy. Lipopolysaccharide, an endotoxin, significantly increased these levels, showing that inflammation is associated with preterm delivery.

Key words: **Uterine smooth muscle cells, ion channel, non-selective cation channel, ATP receptor, gap junction, magnesium.**

Introduction

Electrical excitability of uterine smooth muscle during delivery is relatively high comparing with various other smooth muscles. Uterine contractions are generated by an increase in the concentration of intracellular Ca^{2+} of myometrial cells. The main source of contractile Ca^{2+} in uterine smooth muscle is thought to be Ca^{2+} influx through L-type Ca^{2+} channels rather than release of sequestered calcium from sarcoplasmic reticulum (Kasai et al., 1994; Kwarabayashi, 1997). On the other hand, the regulation of uterine contractile waves is not fully understood and there is a need to search for other sources of contractile Ca^{2+}. Some types of receptor-operated non-selective cation channel (NSCC) have been identified in various smooth muscle cells (Inoue and Isenberg, 1990; Loirand et al., 1991;

Sims, 1992; Vogalis and Sanders 1990; Wang and Large 1991) and have been suggested to be another pathway for Ca^{2+}influx.

The opening of L-type Ca^{2+} channels is regulated by the membrane potential of uterine smooth muscle cells. The membrane potential of smooth muscle is determined by various types of potassium (K) channels. We reported three types of K channels, delayed rectifier K^+ channel, Ca-activated K^+ channel and transient K^+ channel in rat myometrial cells using the whole cell patch-clamp method (Miyoshi et al., 1991). These K^+ channel are found to repolarize the membrane potential after action potentials and terminate muscle contractions. The opening of K^+ channels induces hyperpolarization of the cell to keep the uterine muscle during pregnancy quiescent.

Myometrial NSCCs also play an important role to change the membrane potential of the cell and the depolarization induces spontaneous uterine contractions. We have reported the presence of two types of NSCC, lanthanum (La) sensitive NSCC and purinergic P2X receptors in pregnant rat uterus which are alternative pathways of Ca^{2+} influx (Miyoshi et al., 2004; Miyoshi et al., 2010). NSCCs also induce depolarization of the cell membrane and are possible mechanism for automaticity in the myometrium.

Extracellular magnesium ions (Mg^{2+}) are well known to inhibit uterine contractions and have been reported to suppress the spontaneous depolarization at physiological concentrations. In fact, Mg^{2+} is clinically used to treat uterine contraction of preterm labor. However, the mechanism of Mg^{2+} blockade of uterine contractions is not known. We reported that myometrial NSCCs were blocked at a physiological concentration of external Mg^{2+} and are thought to be targets of Mg^{2+} (Miyoshi et al., 2004; Miyoshi et al., 2010).

Uterine contraction during labor is characterized not only by its automaticity but also by highly synchronized cell contractions in the whole uterus. Cell couplings through gap junction (GJ) channels between myometrial cells are increased dramatically at the end of pregnancy, leading to the onset of labor. This increase has been well established morphologically and functionally (Garfield et al, 1977). We previously reported the change and enhancement of electrical couplings between rat myometrial cells during pregnancy using the double patch-clamp technique (Miyoshi et al., 1996). The enhancement was also observed in a preterm birth model rat treated with antiprogesterone (RU486).

Preterm birth is a major determinant of perinatal morbidity and mortality. (Romero and Mazor, 1988). Inflammatory infiltration of the uterus is known to be one of the causes inducing preterm labor. However, the mechanism of enhancement of uterine contractility in these conditions remains to be elucidated. The Gram-negative bacterial endotoxin, lipopolysaccharide (LPS) is believed to cause adverse pregnancy outcomes (Fidel et al., 1994; Okawa et al., 2001). We have shown that inflammation by LPS treatment on pregnant rat enhanced markedly the level of gene expression of myometrial NSCCs, indicating that increased NSCCs could enhance the excitability of myometrium (Urabe et al., 2009).

Identification of myometrial NSCC currents

The conventional whole-cell patch-clamp method was used in freshly isolated myometrial cells from pregnant rats on day 18-20 of gestation (Miyoshi et al., 2004). NSCC current was isolated by suppressing background K^+ channel currents by tetraethyl-ammonium chloride and L-type Ca^{2+} channel by 5 μM nifedipine. Reduction of external Mg^{2+} to 0.1 mM induced measurable leakage currents. These currents had a linear shape and no time-dependent component as shown in Figure 1. Reduction of sodium ion (Na^+) in the external solution substantially decreased the amplitude of this current, indicating that Na^+ should be the main carrier ion through these channels. This current is blocked by lanthanum (La; blocker of NSCC) ion and Kd was calculated to be 2.4 μM. Also, these channels were observed to have permeability of various monovalent ions and the order of permeability was $K^+ > Cs^+ > Na^+ > Li^+$ (Cs: cesium, Li: lithium). These findings indicate this channel in rat myometrial cells to have the properties of NSCC. The La-sensitive NSCC also had a small but significant conductance for Ca^{2+}, showing that the La-sensitive NSCC may be another pathway for Ca^{2+} leading to uterine contraction.

Possible constituents of La-sensitive NSCC are the transient receptor potential protein (TrpC). The TrpC protein family is suggested to mediate Ca^{2+} entry in myometrial cells. The La-sensitive NSCC have some characteristics similar to TrpC channels such as their ion selectivity and sensitivity to La^{3+}, suggesting the possibility that TrpC serves as a calcium entry pathway in myometrial cells. TrpC-channels, TrpC3 or TrpC4 DNAs were detected in myometrium (unpublished data).

Identification of myometrial P2X7 receptor currents

We found another type of myometrial NSCC current which are induced by external adenosine triphosphate (ATP) in the presence of La^{3+} (Miyoshi et al., 2010). ATP has been reported to enhance the membrane conductance of myometrial cells and uterine contractility. Purinergic P2 receptor

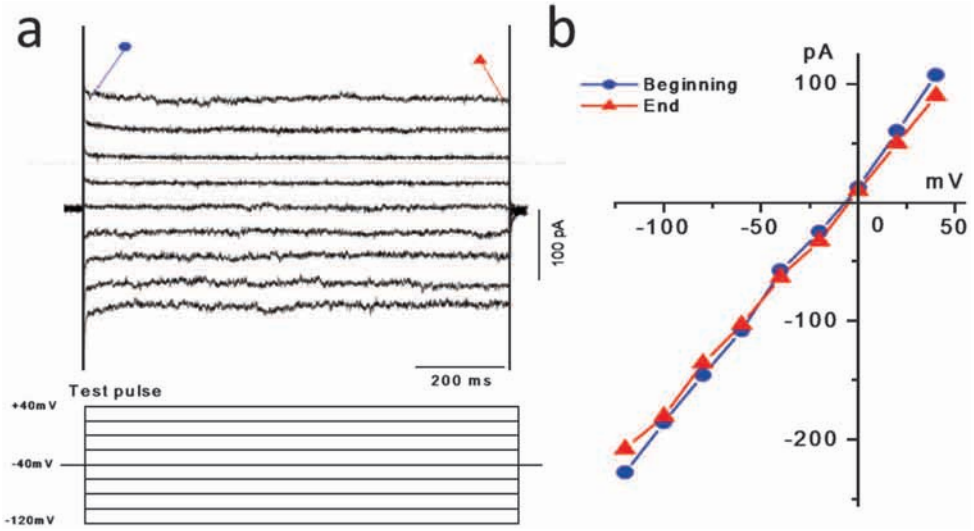

Fig. 1. — Family of NSCC currents obtained from rat myometrial cells and their current-voltage relationship.
a. Superimposed traces of whole-cell currents through NSCC. Note that the currents shown have no time-dependency. Test pulse protocol is shown in the left lower panel. The holding potential was -40 mV. At a membrane potential of -40 mV an inward current was observed. The thin dotted line indicates the zero current level. **b.** The amplitude of the current at the beginning (●) or end (▲) of the test pulse was plotted against the membrane potential. The current-voltage relationships were linear. (H. Miyoshi et al. 2004).

expression has been reported in the myometrium, using molecular biology, but the functional identity of the receptor subtype has not been determined. In our previous study ATP-induced currents were recorded and characterized in single myometrial cells from pregnant rats. Extracellular ATP applied in the range of 10 μM - 1 mM induced currents with an EC_{50} of 74 μM, with no time-dependency, or voltage-dependency as presented in Figure 2. The induced currents carried multiple monovalent cations, with conductances ranked as $K^+ > Cs^+ > Li^+ > Na^+$. They are activated by P2X receptor agonists, with their effectiveness ranked as 2`,3`-O-(4-benzoylbenzoyl)-ATP (Bz-ATP) >> ATP > αβ-methylene ATP (αβ-MeATP) > 2-methylthio ATP (2-MeSATP) ≥ UTP ≥ GTP > ADP. P2X receptor is classified into seven subtypes from P2X1 to P2X7. ATP-induced currents in myometrial cells are not decreased and no desensitization to ATP was observed. These currents are blocked by the selective P2X7 receptor antagonist, 3-(5-(2,3-dichlorophenyl)-1 H-tetrazol-1-yl) methyl pyridine (A438079). We

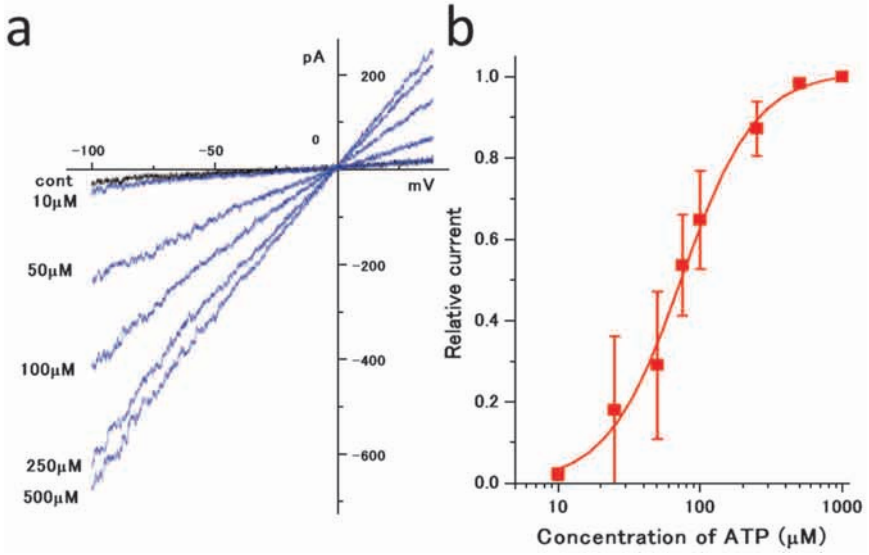

Fig. 2. — Dose-dependency of the currents in response to extracellular ATP.
a. Currents were recorded at ATP concentrations between 10 and 500 μM using the ramp pulse protocol from -100 to +40 mV. The curve labelled "cont" shows a recording in the absence of ATP. Current activation was detected at ATP concentrations as low as 10 μM. **b.** The mean current amplitudes at -80 mV relative to those measured in the presence of 1mM ATP are shown; bars show SDs. EC50 for ATP was estimated to be 74 μM (n = 7) from this curve, which was obtained by fitting the data to the Boltzmann equation. (H. Miyoshi et al. 2010).

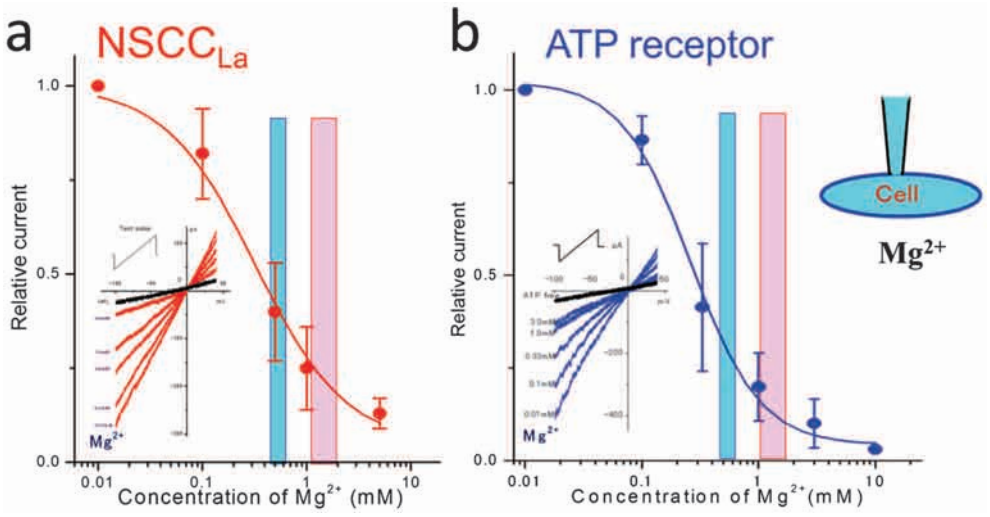

Fig. 3. — Effect of extracellular Mg^{2+} on La-sensitive NSCC and ATP-induced currents.
Effects of Mg^{2+} on the La-sensitive NSCC currents (a: $NSCC_{La}$) and the ATP induced currents (b: ATP receptor) were examined at various Mg^{2+} concentrations. Currents were inhibited by Mg^{2+} in a concentration-dependent manner shown in each small column. Mg^{2+} concentration-effect curves are presented. IC_{50} was calculated to be 0.28 mM for $NSCC_{La}$ in a and 0.26 mM for ATP receptor in b from the curves by fitting the data to the Boltzmann equation. Note that Mg^{2+} did not completely inhibit the current, since 10% of the current persisted at Mg^{2+} concentrations higher than 5 mM. (H. Miyoshi et al. 2004, 2010).

therefore conclude that ATP-induced currents in rat myometrial cells crossed cell membranes via P2X7 receptors.

Blockade of myometrial NSCCs by Mg ion

Consistent with NSCCs of other organs, Mg^{2+} inhibits NSCC currents of rat myometrium in a dose-dependent manner. Extracellular Mg^{2+} inhibits the La-sensitive NSCC currents in a concentration-dependent manner with an IC_{50} value of 0.28 mM as shown in Figure 3a (Miyoshi et al., 2004). We also showed that the P2X7 receptor currents were blocked by Mg^{2+} with an IC_{50} of 0.26 mM as shown in Figure 3b (Miyoshi et al., 2010). These values of IC_{50} are comparable with the physiological concentration of free Mg (Mg^{2+}) in rat serum. In increasing Mg^{2+} from the physiological concentration to the therapeutic range from 1 to 2 mM, the amplitude of myometrial NSCCs current is reduced to around one third. In fact, external application of ATP could induce depolarization of myometrial cells leading to uterine contractions and this depolarization is inhibited significantly by higher concentration of Mg^{2+}. These findings indicate that blocking of myometrial NSCCs is one of mechanism of tocolytic effects by $MgSO_4$.

Clinically, administering extracellular Mg^{2+} is known to inhibit uterine contractions. The observed Mg^{2+} blockade may reasonably explain the inhibitory effect of Mg^{2+} on uterine contractions in the treatment of preterm labor. Further research is needed into the myometrial NSCCs as a therapeutic target in abnormal uterine contraction, as a possible treatment for premature labor.

Enhancement of cell coupling in labor

Gap junction channels play an important role as a pathway of electrical excitation in contraction of the uterus and the regulation of their activities leads to maintenance of uterine quiescence and the onset of labor. In our previous study, the double whole-cell patch-clamp technique was applied to paired cells freshly isolated from pregnant rat myometrium to investigate the functional control during pregnancy (Miyoshi et al., 1996). The macroscopic gap junction currents decayed slowly from an instantaneous, constant-conductance level to a steady-state level described by quasi-symmetrical Boltzmann functions of transjunctional voltage. Unitary junctional conductance was detected to be 85 pS (85-90% of events) and 25 pS under halothane exposure. The properties of gap junction currents in these preparations are almost identical to those of connexin 43. There was no significant difference on the current properties during pregnancy. Junctional conductances as measured in various stages of pregnancy were estimated to be 5 ± 8 nS in preterm, 32 ± 16 nS in term and 7 ± 10 nS in postpartum. The conductance in delivering was six fold greater than that in preterm nondelivering rats and decreased quickly in 24 hours as shown in Figure 4a. An antiprogesterone (RU486) was injected to the rat at the day 18 (D18) to induce preterm labor and gap junctional conductance was measured at D19. The conductance was

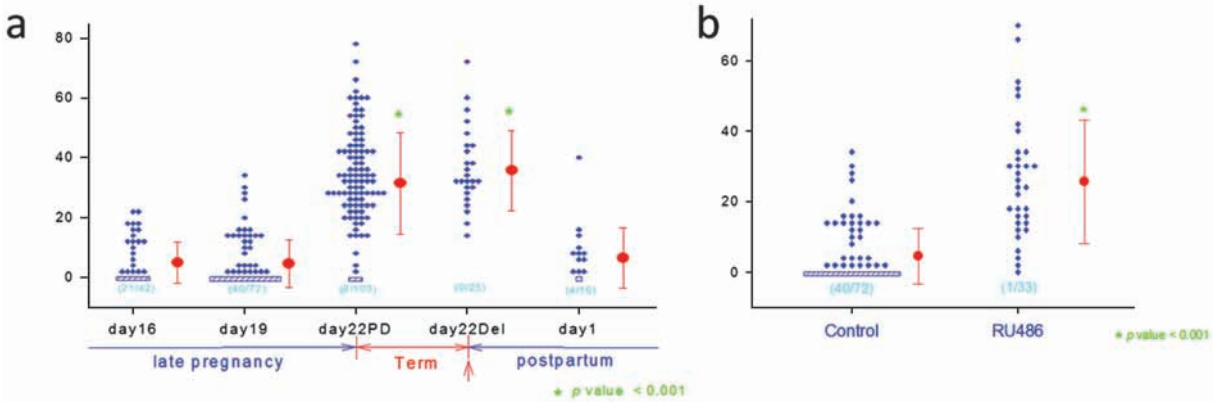

Fig. 4. — Change of junctional conductance during pregnancy.
Individual measurements of junctional conductance from myometrial pairs are plotted for day 16, 19, day 22 predelivery (day22PD), day 22 actively delivering (day22Del), one-day postpartum (PP1) in **a** and day-19 preterm-delivering (RU486) in **b.** Nonzero values are plotted individually as open symbols; for clarity, "zero" values are represented as single open boxes for each group (H. Miyoshi et al. 1996). The mean values of junctional conductance are shown as filled circles (●) with error bars (mean ± SD). Differences among the six groups were significant at the $p < .0001$ level (two-tailed t test).

5 ± 8 nS in control and 26 ± 17 nS in RU486 injected rat, showing that electrical couplings are increased and lead to preterm labor (Fig. 4b). The effect of cyclic AMP dependent agonist on junctional conductance was studied in cell pairs at D22 non delivering. Junctional conductance was decreased to be 78% with isoproterenol of 10 M and 81% with dibutylic c-AMP of 1 mM. We conclude that the enhancement of cell-to-cell couplings at the end of pregnancy and in preterm labor is the increase in numbers of gap junction channels. Junctional conductance is blocked by cyclic AMP dependent agonists.

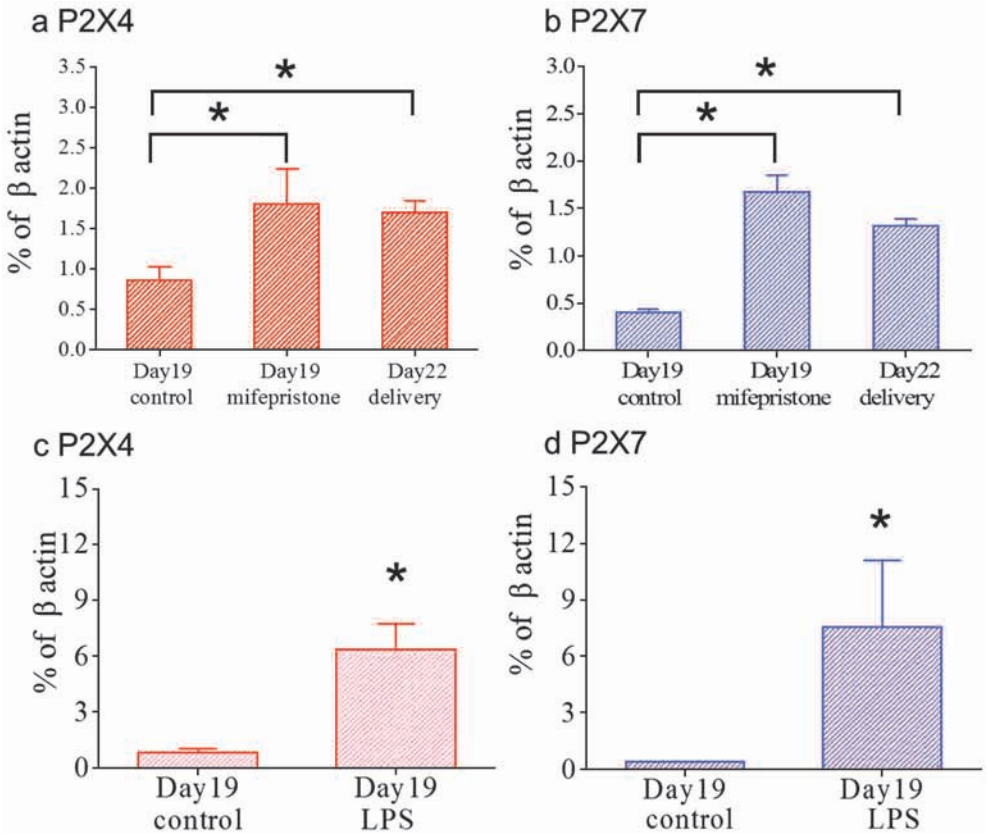

Fig. 5. — Quantitative analysis of P2X4 and P2X7 mRNAs in the myometrium.
a,b) An antiprogesterone mifepristone (RU486) or saline (control; 1 mg for each) was injected subcutaneously at day 18 of gestation. On the next day the uterine tissue was collected. The expression levels of the P2X4 and P2X7 mRNAs were compared with those at delivery on day 22. **c,d)** LPS or saline (C: control; 0.2 mg/kg for each) was injected into the intraperitoneal cavity on day 18 of gestation. The expression levels of the P2X4 and P2X7 mRNAs were shown in **c** and **d** (S. Urabe et al. 2009). The measured data were normalized against those for β-actin. Values are presented as the mean ± S.D (* $P < .01$).

Expression of myometrial NSCCs

We investigated the changes in the expression of myometrial NSCCs during pregnancy and the effect of inflammation in preterm delivery (Urabe et al., 2009). The expression of each subtype of P2X receptor and TrpC channel mRNA was measured by real-time RT-PCR with TaqMan probes (ABI). P2X4 and P2X7 were determined to be dominant subtypes of the P2X channel in the rat myometrium during pregnancy. The expression of P2X4 is increased by 70% in rats delivering on day 22, compared to those delivering on day 15 and that of P2X7 is enhanced by 90% as shown in Figure 5a and 5b. On the other hands, TrpC3 and TrpC4 were detected dominantly. The expression of TrpC3 was increased three times in the late stages of gestation. However, TrpC4 was suppressed by 57% (unpublished data). The mRNA expression of P2X4, P2X7 and TrpC3 channels are increased in the late stages of pregnancy. When the result of the patch-clamp study is considered, the P2X7 channel is suggested to be highly concerned with onset of labor.

The expressions of P2X4 and P2X7 mRNA are enhanced in the preterm model that was treated with antiprogesterone (RU486) at the same level as in labor in comparison to those of the control group as shown in Figure 5a and 5b. This result indicates that these channels are responsible for the hormone-dependent preterm delivery.

As an inflammatory model lipopolysaccharide (LPS; 0.2 mg/kg) was injected into intraperitoneal cavity on day 18 and the myometrium was sampled after six hours. In this model the expressions of P2X4, P2X7 and TrpC3 were enhanced by 7, 18 and 25 times, but TrpC4 was not changed (Fig. 5c and 5d, data for TrpC: unpublished data). The enhancement was much greater than that in normal delivery. This finding therefore suggests that the inflammation in the whole body of the animal may enhance the expression of myometrial NSCCs to accelerate uterine contractility and thereby may induce preterm delivery. The mechanism of preterm delivery with infection may be different from that at the onset of normal delivery at term.

Conclusion

We have revealed various ion channels in myometrial cells concerned with uterine contractility. Regulation of membrane potential of the cell is essential to explain muscle contractility. We present some findings of myometrial NSCCs. However, the role of myometrial NSCCs is still largely unknown and should be the object of further study to clarify the cause of preterm delivery. The expression of some myometrial NSCCs was enhanced remarkably. These NSCCs are likely to be associated with inflammation and may take a part of the cascade of inflammation. New findings on myometrial NSCCs may become the clue to the development of a new treatment for premature birth.

In our studies antiprogesterone was used as a model for preterm delivery. Blocking of progesterone enhanced the expression of myometrial NSCCs and function of gap junctions to increase uterine activities. Although no direct evidence, these findings may reasonably explain the inhibition of preterm birth by progesterone treatment.

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