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Mechanisms of galanin-induced contraction in the rat myometrium

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1 A neuropeptide, galanin, regulates the reproductive process and directly induces myometrial contraction. The aim of this study was to determine the mechanism of galanin-induced myometrial contraction. For this purpose, we simultaneously measured intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and tension using fura-PE3-fluorometry and the rat longitudinal myometrium. The effect of galanin on the Ca^{2+} sensitivity of the contractile apparatus was examined in β-escin permeabilized strips. The expression of galanin and the galanin receptors mRNAs in the rat myometrium were determined by reverse transcription polymerase chain reaction (RT-PCR).

2 Galanin (10–300 nM) induced phasic contraction with or without oscillation in the pregnant rat myometrium in a concentration-dependent manner. The maximal response was obtained at 100 nM. There was no significant difference either in the maximal responses or EC_{50} values for galanin-induced myometrial contractions among myometriums from non-pregnant and pregnant (day 4, day 11, day 20, day 22) rats.

3 In the day 20 and 22 pregnant myometriums, assigning the levels of $[Ca^{2+}]_i$ and tension at 40 mM K^+-depolarization to be 100%, galanin increased the $[Ca^{2+}]_i$ and tension to $126.9 \pm 2.9\%$ and $116.3 \pm 2.7\%$, respectively. Diltiazem (10 μM) inhibited the galanin-induced elevation of $[Ca^{2+}]_i$ and tension to $71.9 \pm 2.4\%$ and $16.2 \pm 0.7\%$, respectively. Ni^{2+}, by itself, decreased the basal $[Ca^{2+}]_i$ to $-50.2 \pm 3.9\%$ without affecting resting tension. After Ni^{2+} treatment, galanin-induced increases in $[Ca^{2+}]_i$ and tension were $-19.6 \pm 3.4\%$ and $0.9 \pm 0.1\%$, respectively. In myometrium treated with diltiazem, no oscillation in $[Ca^{2+}]_i$ and tension was observed. In Ca^{2+}-free solution with 0.1 mM EGTA, galanin increased $[Ca^{2+}]_i$ from $-40.2 \pm 2.7\%$ to $-18.0 \pm 2.6\%$ and induced transient contraction ($3.6 \pm 0.8\%$).

4 In β-escin permeabilized myometrium, galanin enhanced the contraction induced by 0.3 μM Ca^{2+} in the presence of GTP. In the presence of GDPβS (1 mM) instead of GTP, galanin failed to increase the Ca^{2+} sensitivity of the contractile apparatus.

5 RT-PCR revealed that galanin mRNA was hardly expressed in the non-pregnant rat myometrium and increased to reach a maximal level at mid pregnancy (day 11), but decreased to the same level as in the non-pregnant myometrium at term (day 22). Type 2 galanin receptor (GALR2) mRNA was found to be expressed in the rat myometrium whereas type 1 galanin receptor (GALR1) mRNA expression was not detected.

6 In conclusion, galanin induces contraction of the rat myometrium by increasing $[Ca^{2+}]_i$ as well as by increasing Ca^{2+} sensitivity of the contractile apparatus. Galanin-induced increases in $[Ca^{2+}]_i$ are caused by both intracellular Ca^{2+} release and Ca^{2+} influx from extracellular space. The responsiveness of the rat myometrium to galanin does not change during pregnancy. The galanin mRNA is expressed in the rat myometrium and it is upregulated during mid-pregnancy. Rat myometrium expresses GALR2 but not GALR1 mRNA. By changing mRNA expression in the myometrium during pregnancy, galanin may act as a paracrine or autocrine mediator in the regulation of myometrial contractility.

Keywords: Galanin; uterine contraction; intracellular Ca^{2+} concentration; skinned fiber; reverse transcription polymerase chain reaction

Introduction

Galanin is a neuropeptide containing 29 amino acids (30 amino acids in humans) originally isolated from porcine small intestine and found to contract gastrointestinal smooth muscle (Tatemoto *et al*., 1983). Subsequent studies revealed that galanin is widely distributed in the central nervous system, peripheral nerves and several endocrine tissues and that it has a broad spectrum of biological effects, such as hormone and neurotransmitter release, antinociception, change in feeding behavior and smooth muscle contraction or relaxation (Rokaeus, 1987). These biological effects of galanin are mediated by receptors coupled with pertussis toxin-sensitive guanosine 5′-triphosphate binding protein (G-protein) to downstream effector systems, including adenylate cyclase, phospholipase C (PLC) and receptor-activated ion channels (de Mazancourt *et al*., 1994; Gillison & Sharp, 1994; McLeod *et al*., 1994; Valkna *et al*., 1995). Recently, two subtypes of galanin receptor have been cloned and termed GALR1 and GALR2, respectively (Habert-Ortoli *et al*., 1994; Burgevin *et al*., 1995; Parker *et al*., 1995; Howard *et al*., 1997).

It was reported that galanin plays an important role in the regulation of reproductive processes. Galanin stimulates gonadotropin-releasing hormone and luteinizing hormone release in the hypothalamus and pituitary thereby increasing estradiol, progesterone and androstenedione production in the ovary, and decreasing metalloproteinase-inhibitor activity in the ovary (Cheung *et al*., 1996). Avian galanin evokes oviposition through mechanisms of the induction of uterine contraction (Li *et al*., 1996). In mammalian species, some studies have shown that galanin itself induces myometrial contraction (Bek *et al*., 1988; Stjernquist *et al*., 1988; Shew *et al*., 1992). However, the mechanisms of the galanin-induced myometrial contraction has not been characterized in detail, especially concerning its intracellular signaling pathway.

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Concerning the mode of action, galanin may be a local hormone regulating myometrial contractility, because galanin mRNA was detected in the endometrial stromal cells (Vrontakis *et al*., 1993) and galanin-like immunoreactivity has been shown to exist in myometrial nerves throughout the uterine horns and cervix in the rat (Shew *et al*., 1992). However, the mRNA expressions of galanin and the two galanin receptor subtypes in the myometrium has not yet been assessed.

In the present study, the mechanism underlying the galanin-induced rat myometrium contraction was elucidated by simultaneous measurements of the intracellular Ca^{2+} ($[Ca^{2+}]_i$) and tension of intact fura-PE3-loaded myometrial strips and by tension measurement under the clamped $[Ca^{2+}]_i$ level using β-escin permeabilized strips. Using reverse transcription and polymerase chain reaction (RT-PCR), the expression of galanin mRNA at the various gestational stages and GALR1 and GALR2 mRNAs were determined in the rat myometrium.

Methods

Tissue preparation

The study protocol was approved by the Animal Care and Committee of Research Institute of Angiocardiology, Faculty of Medicine, Kyushu University. Virgin female Wister-Kyoto rats weighing 200–250 g were housed under a 13 h light, 11 h dark photoperiod, with lights on at 7. 00 a.m. The female rats were paired overnight with males and the morning on which a vaginal plug was detected was taken as day 1 of pregnancy. Rats were sacrificed with ether at different intervals after mating. Cyclic virgin rats were also sacrificed. Bilateral uterine horns were excised and placed in a physiological saline solution (PSS) consisting of the following compositions (in mM): NaCl 123, KCl 4.7, $CaCl_2$ 1.25, $MgCl_2$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 15.5, and D-glucose 11.5, gassed with 95% O_2 and 5% CO_2. The myometrium from the middle part of the horn was dissected in a longitudinal direction, visible connective tissue and vessels were removed, and then the myometrium was cut into strips measuring 1.5 mm in width and 3.5 mm in length for the following measurements.

Tension measurement of intact myometrial strips

The myometrial strips were mounted vertically in a quartz organ bath and connected to a force transducer (TB-612T, Nihon Koden, Japan). The strips were stimulated with 40 mM K^+ PSS, which was made by an equimolar substitution of KCl for NaCl, every 15 min with stepwise increase in resting load until the maximal response was obtained. The resting load so obtained was around 50 mg. The developed tension was expressed as a percentage, while assigning the values in normal (5.9 mM K^+) and 40 mM K^+ PSS to be 0% and 100%, respectively.

Fura-PE3 loading and measurement of $[Ca^{2+}]_i$

The myometrial strips from the pregnant rats were loaded with fura-PE3 in the form of acetoxymethyl ester (fura-PE3/AM) by incubation in Dulbecco's-modified Eagle's medium containing 40 μM fura-PE3/AM dissolved in dimethylsulphoxide and 5% fetal bovine serum for 6 h at 37°C (containing 0.03% Pluronic F-127). Subsequently, the strips were washed with PSS containing 1.25 mM Ca^{2+} at 37°C to remove the dye from the extracellular space and then were equilibrated in normal PSS for at least 1 h before the initiation of measurements.

The changes in the fluorescence intensity of the fura-PE3-Ca^{2+} complex were simultaneously monitored during the measurement of the force, using front-surface fluorometry, as previously reported (Abe *et al*., 1990). The fluorescence intensity was measured by a fluorometer, which was specially designed for fura-2 fluorometry (CAM-OF-1). The strips were illuminated with alternating (400 Hz) excitation lights (340 and 380 nm). The fluorescence (500 nm) intensities at 340 nm (F340) and 380 nm (F380) excitation, their ratio (F340/F380) and the tension development were continuously measured. These data were stored in a Macintosh computer using a data acquisition system (MacLab). The fluorescence ratio (F340/F380), which indicates $[Ca^{2+}]_i$, was expressed as a percentage, assigning the values in normal (5.9 mM K^+) and 40 mM K^+ PSS to be 0 and 100%, respectively. All simultaneous measurements of $[Ca^{2+}]_i$ and force were performed at 37°C.

Tension measurement of β-escin permeabilized myometrial strips

Permeabilization of rat myometrial strip was done according to the methods described by Kobayashi *et al*. (1989) with minor modifications. The small strips (about 0.5 mm in width and 2 mm in length) were mounted between two tungsten wires, one of which was fixed and the other one was attached to a force transducer (UL2; Minebea Co., Japan). After measuring steady contractions induced by 40 mM K^+ in normal external solution, the strips were incubated in relaxing solution containing 100 mM potassium methanesulphonate, 2.2 mM Na_2ATP, 3.38 mM $MgCl_2$, 10 mM creatine phosphate, 20 mM Tris-maleate (pH = 6.8), 2 μM calmodulin and 2 mM ethyleneglycol-bis(β-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA) for several minutes and then were treated for 60 min with 40 μM β-escin and 10 μM Ca^{2+} buffered with 10 mM EGTA. The composition of Ca^{2+} solution (activating solution) was the same as the relaxation solution described above, except that it contained the indicated concentration of free Ca^{2+} buffered by 10 mM EGTA. Intracellular Ca^{2+} store was depleted by 1 μM ionomycin in the relaxing solution after permeabilization. Experiments using permeabilized tissue were performed at room temperature. The resting tension in relaxing solution and maximal tension induced by 10 μM Ca^{2+} were taken as 0% and 100%, respectively.

RT-PCR

Myometrial and ileal total RNAs were isolated by the guanidinium thiocyanatephenol-chloroform method (Chomczynski & Sacchi, 1987). During the tissue trimming of the myometrial smooth muscle cells, care was taken not to include the connective tissue and vessels. Total RNAs were digested by RNase free DNase to exclude the possible contamination by genomic DNA. The amount of the total RNA was determined using a spectrophotometer.

The primers for rat galanin, rat GALR1 and GALR2 were designed according to the published sequences by Vrontakis *et al*. (1993), Parker *et al*. (1995) and Howard *et al*. (1997), respectively. For the control, we also performed RT-PCR for β-actin, using primers reported in our previous study (Niiro *et al*., 1997). PCR primers and RT primer for galanin were 5′-ATGCCAACAAAGGAGAAGAG-3′ (76–95), 5′-AGGTGCAAGAAACTGAGAAA-3′ (280–299), and 5′-CTAGGTCTTCTGAGGAGGTG-3′ (345–364), respectively. The expected size of the PCR product for rat galanin was 224 base

pair (bp) and the product should be digested into 145 bp and 79 bp fragments by Mbo I. PCR primers and RT primer for GALR1 were 5′-GGCGTGGGCTTCATCTGG-3′ (460–479), 5′-CACCGAGGAGTTGCTGTATG-3′ (869–888) and 5′-AGAGAAAGGCGTGATGATG-3′ (894–913), respectively. The expected size of the PCR product for GALR1 is 429 bp and should be digested into 232 bp and 197 bp fragments by *Eco*T14I. PCR primers and RT primer for GALR2 were 5′-TGGCCGACCTGTGTTTCATC-3′ (206–225), 5′-GGCCG-GAGAAGAGCAGTGC-3′ (457–476) and 5′-TACGCGCA-TAGGTCAGACTG-3′ (609–628), respectively. The expected sizes of PCR products for GALR2 and β-actin are 271 bp and 224 bp, respectively.

RT-PCR was performed as previously reported (Niiro *et al*., 1997). In brief, the total RNA (1 μg) was incubated for 60 min at 37°C for RT reaction in a total volume of 20 μl. An aliquot (1 μl) of RT product was used for PCR amplification in a total volume of 11 μl. The thermal cycle profile used in this study was (1) denaturing for 30 s at 94°C, (2) annealing primers for 90 s at 55°C, (3) extending the primers for 30 s at 72°C. The PCR amplifications were performed for 35 cycles. In the case of GALR1 and GALR2, annealing was carried out for 60 s at 60°C. We also performed RT-PCR for β-actin as a control. PCR amplification for β-actin was carried out for 25 cycles as previously reported. A portion (10 μl) of the PCR mixture was electrophoresed in 3% agarose gel in TAE buffer. The gel was stained with ethidium bromide and then photographed. For the quantitative analysis of RT-PCR products, the density of bands for myometrial mRNA was determined by a scanning densitometry system (CS9000, Shimadzu, Tokyo, Japan), then the levels of PCR products were normalized to PCR products for β-actin. The direct sequencing of the PCR product for GALR2 was performed using an Autosequencer (373S, ABI, Foster City, CA, U.S.A.) according to the manufacturer's instruction.

Drugs

Rat galanin was purchased from the Peptide Institute (Osaka, Japan). Diltiazem and β-escin were from Sigma (St. Louis, MO. U.S.A.). Fura PE3/AM and Pluronic F127 were from Texas Fluorescence Laboratory (Austin, TX, U.S.A.). Bovine brain calmodulin was from Seikagaku Corporation (Tokyo, Japan). GDPβS and GTPγS were from BIOMOL Research Laboratories (Plymouth Meeting, PA, U.S.A.) and Boehringer Mannheim (Mannheim, Germany), respectively. M-MLV (Moloney Murine Leukemia Virus) reverse transcriptase was purchased from BRL (Gaithersburg, MD, U.S.A.). NuSieve™ 3:1 agarose was from TaKaRa (Kyoto, Japan). RNase inhibitor and $\phi \times 174/$*Hinc*II digest were purchased from TOYOBO (Osaka, Japan). Taq DNA polymerase was from Pharmacia Biotech. (Uppsala, Sweden). Oligonucleotides for primers were synthesized by Sawady Technology Inc. (Tokyo, Japan). All other chemicals were of the highest grade commercially available.

Statistical analysis

All data are expressed as mean ± standard errors (s.e.m.) along with the number of observations ($=n$). Student's *t*-test was used to determine statistical difference between two mean values. Bonferroni/Dunn's analysis was used to determine statistical significance between the five groups in Table 1 and Figure 6c. P values <0.05 were considered to be statistically significant. The four-parameter logistic model was used to fit the sigmoidal curve to the concentration response of a drug.

Table 1 EC_{50} values and maximal responses of galanin-induced tension development of the rat myometrium from different gestational stages

	EC_{50} values (nM)	*Max. cont.* (%)	n
Non-pregnant	34.5 ± 8.0	95.9 ± 4.5	4
Day 4	35.2 ± 12.3	88.5 ± 3.9	5
Day 11	41.3 ± 5.9	87.4 ± 3.5	5
Day 20	36.4 ± 2.7	95.9 ± 4.4	6
Day 22	26.1 ± 2.2	107.0 ± 7.5	5

All data are expressed as the means ± s.e.m. obtained from different animals ($n \geqslant 4$). There is no significant difference in either EC_{50} value or maximal response among the five groups when analysed by Bonferroni/Dunn's analysis.

Results

Variation of the galanin-induced rat myometrial contraction and its change during pregnancy

Application of galanin (100 nM) in the normal PSS induced a phasic contraction, namely, a rapid and transient increase in tension with or without oscillation in the rat myometrial strips. Oscillatory tension continued for more than 20 min in some preparations (Figure 1a), whereas it disappeared after several times of repetition (Figure 1b) or there was no repetition (Figure 1c). For example, in rats on days 20 to 22 of pregnancy, six of 15 (40%) myometrium showed oscillatory contraction, and nine of 15 (60%) induced only transient contraction without oscillation. Therefore, we plotted the initial peak value of each contraction irrespective of whether or not it was followed by oscillation and constructed dose-response curves for galanin-induced myometrial contraction. As shown in Figure 2, galanin (1–300 nM) caused dose-dependent contraction of the myometrium on day 20 of gestation. Maximal response was obtained at 100 nM and EC_{50} value was 36.4 ± 2.7 nM ($n=6$). Similar experiments were performed using myometrium obtained from non-pregnant rats and pregnant rats at different stages (days 4, 11, 22) of gestation. Maximal response and the EC_{50} value at each gestational stage are shown in Table 1. There were no significant differences in either EC_{50} values or maximal responses among the various stages of pregnancy.

Effect of diltiazem and Ni^{2+} on the galanin-induced increases in the $[Ca^{2+}]_i$ and tension of the pregnant rat myometrium

We used myometrium on day 20 and 22 of gestation for the simultaneous measurements of $[Ca^{2+}]_i$ and tension. Figure 3a shows one of the recordings of the changes in $[Ca^{2+}]_i$ and tension induced by 100 nM galanin in normal PSS. Galanin induced rapid and transient rises both in $[Ca^{2+}]_i$ and tension, which reached a peak level within 1 min, followed by a gradual decline to the preapplication levels. In this case and some other cases, oscillatory changes in the $[Ca^{2+}]_i$ and tension were superimposed on the declining phase of the initial transient elevation of $[Ca^{2+}]_i$ and tension. In attempt to clarify the Ca^{2+} influx pathway, diltiazem (an L-type Ca^{2+} channel blocker) or Ni^{2+} (a non-selective Ca^{2+} channel blocker) at the minimum concentrations to completely inhibit 40 mM K^+-induced increases in $[Ca^{2+}]_i$ and tension, was applied during activation by galanin. As shown in Figure 3b, pretreatment with 10 μM diltiazem significantly inhibited the galanin-induced increases in $[Ca^{2+}]_i$ and tension. The pretreatment by Ni^{2+} inhibited galanin-induced increases in $[Ca^{2+}]_i$ and tension more

effectively than diltiazem did. However, still small transient increases in $[Ca^{2+}]_i$ and tension remained, indicating that galanin induces not only Ca^{2+} influx but also intracellular Ca^{2+} release. Figure 3d summarizes the results obtained from a number of experiments performed in a similar manner as those represented in Figure 3a–c. The treatment with diltiazem completely inhibited the occurrence of the oscillations in $[Ca^{2+}]_i$ and tension ($n=8$). In addition, galanin exclusively induced oscillations in the presence of 2.5 mM external Ca^{2+} in the strips, in which only transient contractions were observed in the 1.25 mM external Ca^{2+} ($n=5$, data not shown).

Effect of the removal of extracellular Ca^{2+} with EGTA on the galanin-induced increases in the $[Ca^{2+}]_i$ and tension of the pregnant rat myometrium

To examine the effect of galanin on the Ca^{2+} release from intracellular store, we measured galanin-induced changes in $[Ca^{2+}]_i$ and tension in Ca^{2+}-free PSS containing 0.1 mM EGTA. When the strip was exposed to the Ca^{2+}-free PSS containing 0.1 mM EGTA, $[Ca^{2+}]_i$ decreased to $-40.2\pm2.7\%$ ($n=10$) in 1 min without affecting the resting tension. At this point depolarization with 40 mM K^+ did not induce the increase in either $[Ca^{2+}]_i$ or tension, indicating extracellular Ca^{2+} was completely chelated by EGTA, and there was no Ca^{2+}-influx (data not shown). In a Ca^{2+}-free PSS containing 0.1 mM EGTA, galanin induced a small $[Ca^{2+}]_i$ transient ($-18.0\pm2.6\%$, $n=10$) accompanied by a small transient contraction ($3.6\pm0.8\%$, $n=10$) (Figure 4a). When the strip was treated with Ca^{2+}-free PSS with higher concentration of EGTA (0.5 mM), galanin failed to induce elevations of either $[Ca^{2+}]_i$ or tension (Figure 4b). In Ca^{2+}-free PSS with 0.5 mM EGTA, however, 100 nM oxytocin induced $[Ca^{2+}]_i$ transient associated with transient tension (Figure 4c).

Effect of galanin on the Ca^{2+} sensitivity of the contractile apparatus in the β-escin permeabilized rat myometrium

As shown in Figure 5a, the application of the solution containing submaximal concentration of Ca^{2+} (0.3 μM) and 10 μM GTP induced tension development, which reached a steady level. Addition of 100 nM galanin induced a small enhancement of tension in the presence of 0.3 μM Ca^{2+}. Subsequent application of 10 μM GTPγS, a non-hydrolyzable GTP analog, induced further Ca^{2+} sensitization, indicating that permeabilized myometrial strip maintained G-protein coupled signal transduction. Compared with the GTPγS-induced Ca^{2+} sensitization, galanin-induced Ca^{2+} sensitization was much smaller. In contrast, both galanin- and GTPγS-induced enhancement of Ca^{2+}-contraction could be abolished by the presence of 1 mM GDPβS, a nonhydrolyzable GDP analog (Figure 5b).

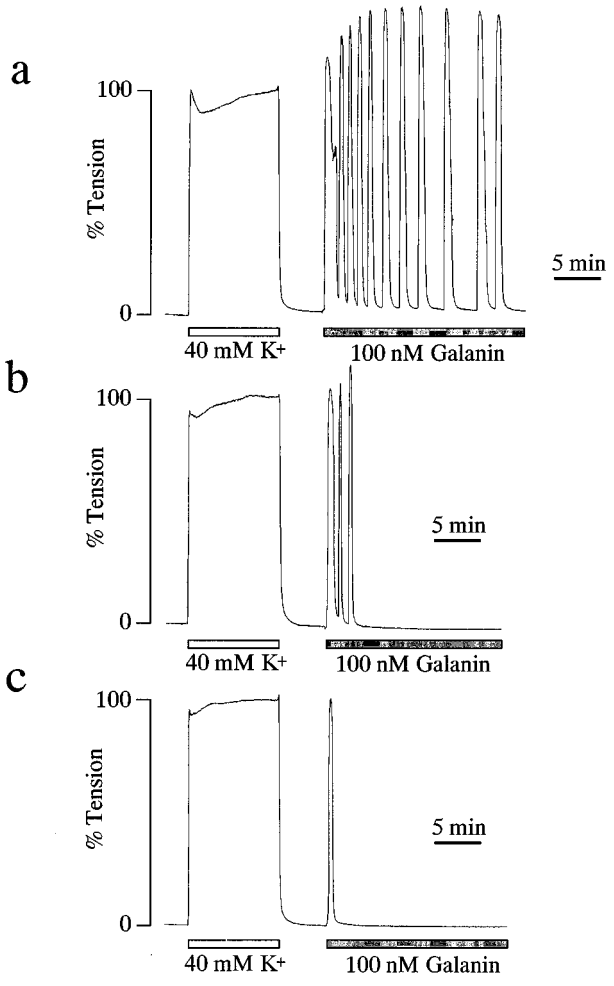

Figure 1 Representative recordings of various types of tension development by galanin in the day 20 pregnant rat myometrium in the normal PSS. Galanin (100 nM) was applied to day 20 pregnant rat myometrium in the normal PSS. (a) Galanin induced a rapid and transient increase in tension, followed by oscillation lasting more than 20 min. (b) Galanin induced a rapid and transient increase in tension and subsequent oscillation. In this case after two subsequent episodes of oscillatory tension, the tension returned to the resting level. (c) Galanin induced only a transient increase in tension. The developed tension was expressed as a percentage, assigning the values in normal (5.9 mM K^+) and 40 mM K^+ PSS to be 0% and 100%, respectively.

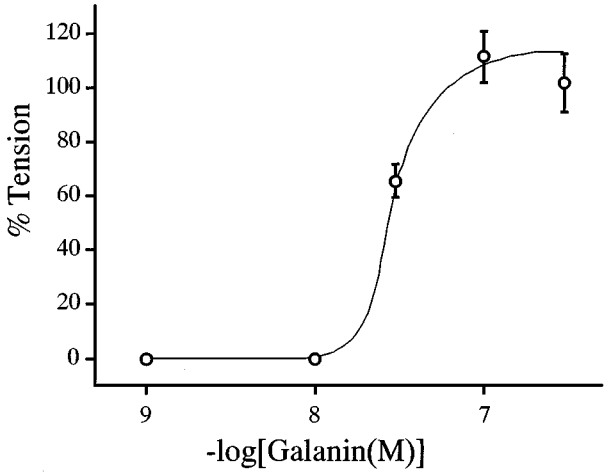

Figure 2 Concentration-response relation of galanin-induced rat myometrial contraction. The peak level of the tension development of day 20 pregnant rat myometrium induced by different concentrations (1–300 nM) of galanin was plotted. Each symbol shows the mean value of the galanin-induced developed tension from six different rats. The tension was expressed as a percentage, assigning the values in normal (5.9 mM K^+) 40 mM K^+ PSS to be 0 and 100%, respectively. Vertical bars represent s.e.m.

Change in the expression of galanin mRNA in the rat myometrium during pregnancy

Figure 6a shows the expression of galanin mRNA in the rat myometrium, determined by RT-PCR. The apparent PCR product of the expected size could be detected in the myometrium on day 11 of gestation, while it could not be detected in the non-pregnant myometrium or in the myometrium on day 22 under the present PCR condition. In the myometrium of day 4 and day 20 of gestation, faint bands could be detected. This PCR product was digested into 145 bp and 79 bp fragments by Mbo I as predicted (Figure 6b), indicating that the PCR product derived from rat galanin mRNA. On the other hand, the bands for β-actin were detected clearly and equally in all the specimens. To further confirm these changes in mRNA expression of galanin, we repeated a similar experiment on three different animals for each gestational stage. Figure 6c shows the mean values and s.e.m of the ratios (galanin/β-actin) as determined by the densitometer and the negative films of the photographs, in order to estimate the relative expression level of galanin mRNA as compared with the expression of β-actin mRNA. Galanin mRNA was most abundantly expressed at mid gestation, and then decreased and returned to the level similar to the non-pregnant myometrium at term.

Expression of GALR1 and GALR2 mRNA in the rat myometrium and ileum

Using total RNA prepared from rat longitudinal myometrium and specific primers designed for GALR1 and GALR2 based on the published sequences, the expression of the two galanin receptor subtypes were examined by RT-PCR. For comparison, the expression of these receptors in the rat ileum was also determined. As shown in Figure 7a, GALR1 mRNA was detected in ileum but not in the myometrium, while β-actin mRNA was clearly detected in both tissue specimens. The GALR1 band was not detected in the myometrium even when the PCR amplification was increased to 45 cycles (data not shown). The PCR product for GALR1 was digested into fragments of 232 bp and 197 bp by *Eco*T14I as predicted (Figure 7b). In contrast, the bands of PCR product for GALR2 were detected in both ileum and myometrium (Figure 7c). When the reverse transcriptase had been omitted, the expected size of band was not detected (Figure 7d), indicating the PCR product was not derived from the genomic DNA. Direct sequencing of the PCR product showed that it was identical to the published sequence of GALR2 mRNA. These data shows that rat longitudinal myometrial smooth muscle cells possesses only GALR2.

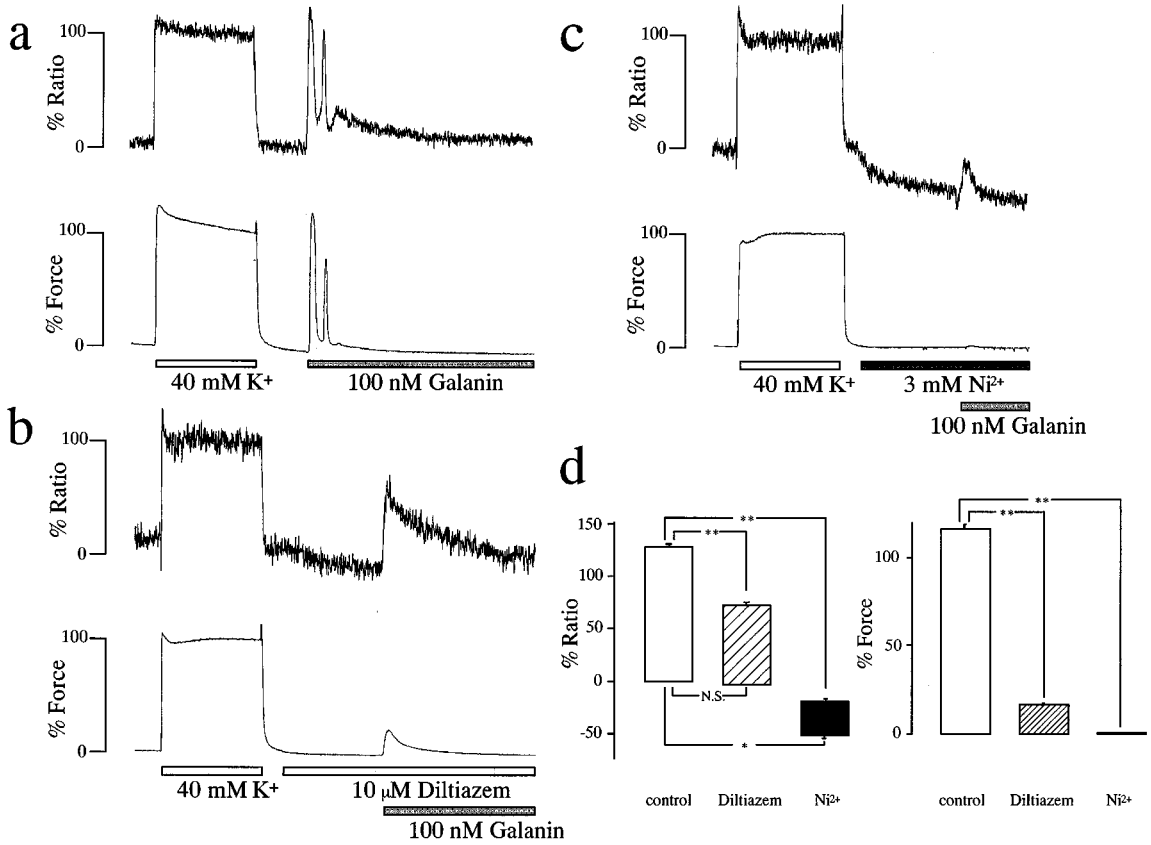

Figure 3 Effects of diltiazem and Ni^{2+} on the galanin-induced changes in $[Ca^{2+}]_i$ and tension in the pregnant rat myometrium. (a) Representative recording showing the effect of Galanin (100 nM) on the $[Ca^{2+}]_i$ (upper trace) and tension (lower trace) of the pregnant rat myometrium in the normal PSS. (b) Representative recording showing the effect of 10 μM diltiazem on the galanin-induced increase in $[Ca^{2+}]_i$ and tension. Diltiazem was applied 10 min before galanin administration. (c) Representative recording showing effect of 3 mM Ni^{2+} on the galanin-induced increase in $[Ca^{2+}]_i$ and tension. Ni^{2+} was applied 10 min before galanin administration. (d) Summary of the inhibitory effect of diltiazem and Ni^{2+} on the galanin-induced increases in $[Ca^{2+}]_i$ and tension of the pregnant rat myometrium. Each value shows the mean of different animals. The developed tension and $[Ca^{2+}]_i$ were expressed as a percentage, assigning the values in normal (5.9 mM K^+) and 40 mM K^+ PSS to be 0 and 100%, respectively. All data are expressed as the means$\pm$s.e.m ($n=6-15$). $^*P<0.05$, $^{**}P<0.01$ as compared with control. N.S.; not significant.

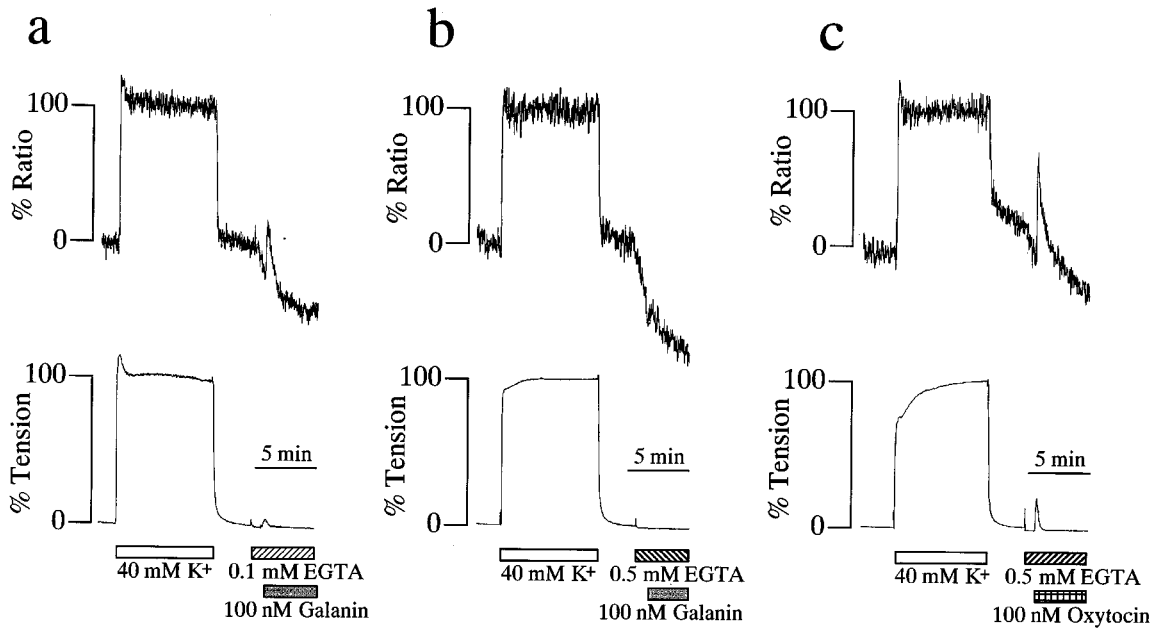

Figure 4 Effect of galanin and oxytocin on the $[Ca^{2+}]_i$ and tension in the rat myometrium in the Ca^{2+}-free PSS containing EGTA. Representative recording showing the effect of 100 nM galanin on the $[Ca^{2+}]_i$ and tension of the pregnant rat myometrium in the Ca^{2+}-free PSS containing (a) 0.1 mM, or (b) 0.5 mM EGTA. (c) Representative recording showing the effect of 100 nM oxytocin on the $[Ca^{2+}]_i$ and tension of the pregnant rat myometrium in the Ca^{2+}-free PSS with 0.5 mM EGTA. The strips were treated for 1 min with Ca^{2+}-free PSS with EGTA before either galanin or oxytocin was added. The developed tension and $[Ca^{2+}]_i$ were expressed as a percentage, assigning the values in normal (5.9 mM K^+) and 40 mM K^+PSS to be 0 and 100%, respectively.

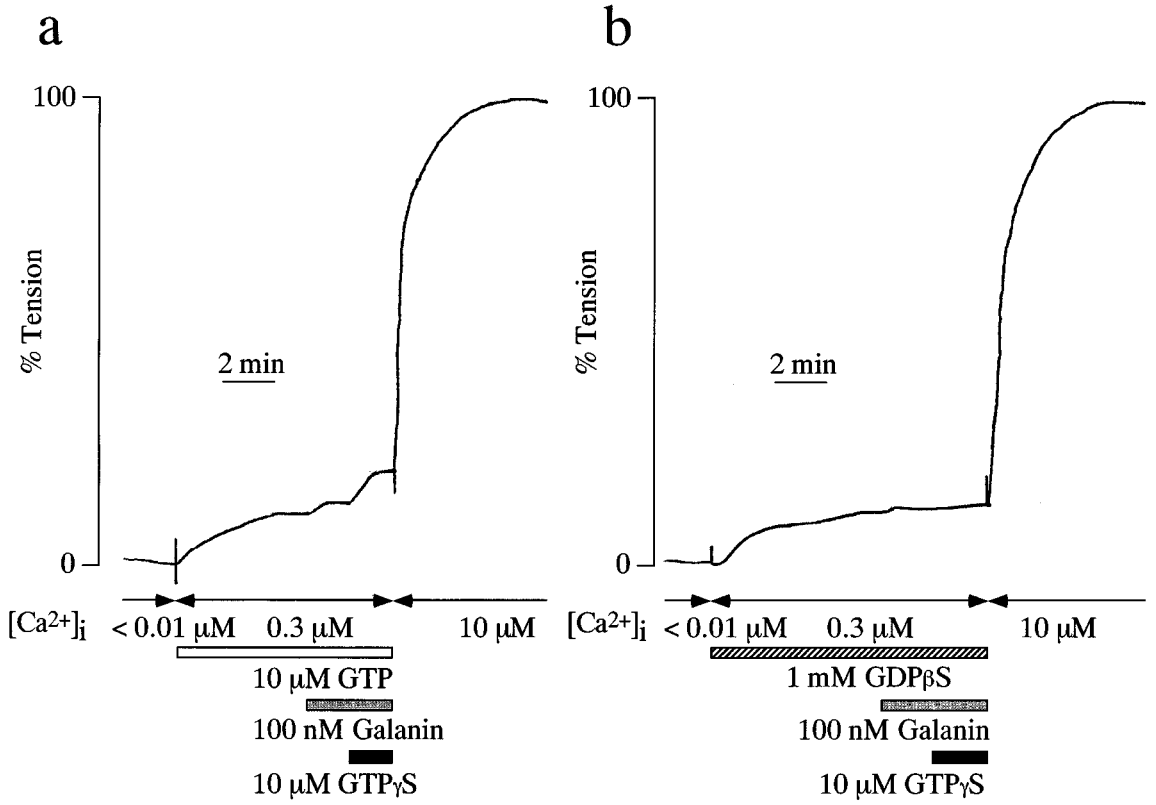

Figure 5 Effect of galanin on the Ca^{2+}-induced contraction in the β-escin permeabilized rat myometrium. (a) After the permeabilization with 40 μM β-escin and depletion of the intracellular Ca^{2+} store with 1 μM ionomycin, the rat myometrial strip was contracted by 0.3 μM Ca^{2+} in the presence of either 10 μM GTP, or (b) 1 mM GDPβS. When the tension reached steady state, 100 nM galanin was added. GTPγS (10 μM) was applied when the tension reached at the plateau level after the galanin administration. Maximal tension was obtained in the presence of 10 μM Ca^{2+}. The developed tension was expressed as a percentage, assigning the values in the relaxing solution ($[Ca^{2+}]_i < 0.01$ μM) and in the activating solution ($[Ca^{2+}]_i = 10$ μM) to be 0 and 100%, respectively.

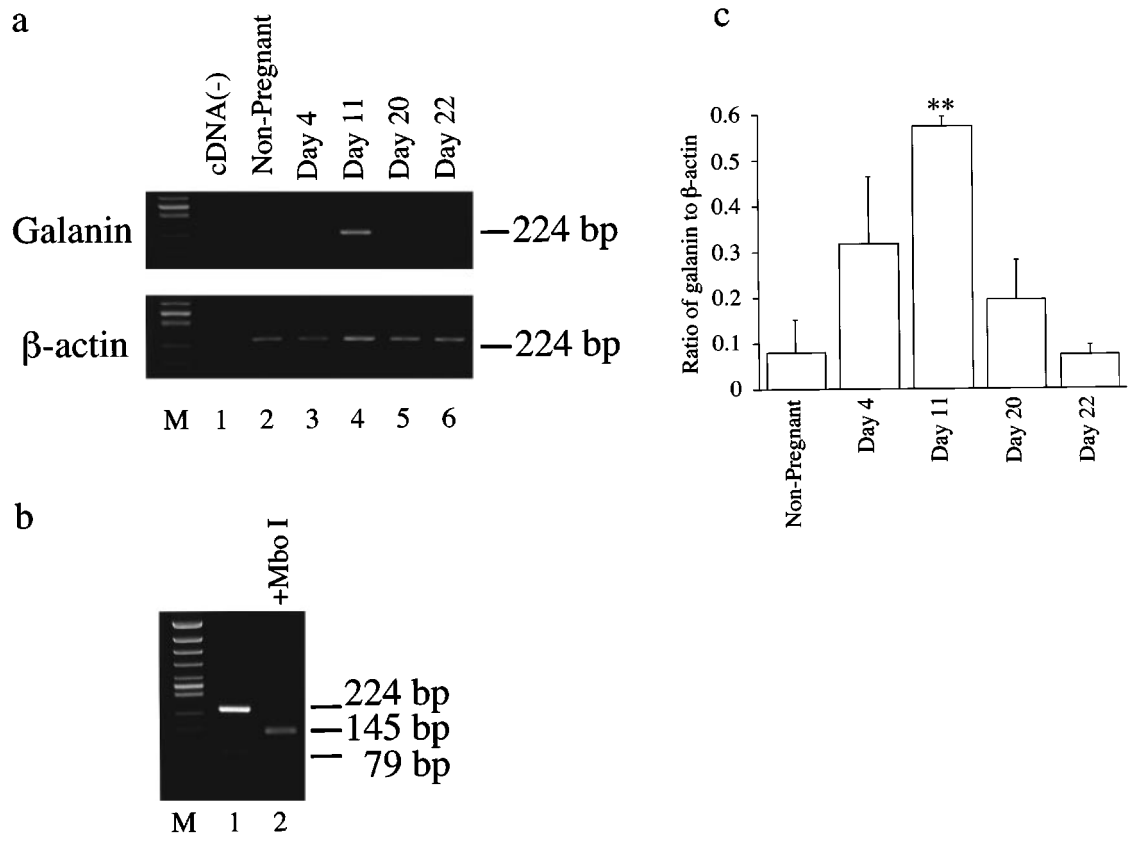

Figure 6 Detection of galanin mRNA by RT-PCR in the non-pregnant and pregnant rat myometrium. (a) Representative photographs showing the expression of galanin (upper) and β-actin (lower) mRNA in the rat myometrium during pregnancy. RT-PCR was performed using the total RNA from non-pregnant and pregnant rats. PCR amplifications for galanin and β-actin were performed at 35 and 25 cycles, respectively. The predicted sizes of each PCR product is shown beside the photographs. Lane 1 represents a negative control, where the cDNA template had been omitted. Lane M represents the DNA size marker (ϕX174/*Hinc*II digest). The size of each band is, from top to bottom, 392, 345+341+335, 297+291, 210 and 162 bp. (b) Identification of the PCR product for galanin by digestion with the restriction enzymes. Lane 1, the PCR product for rat GALR1 without digestion. Lane 2, the PCR product for rat galanin as digested by *Mbo*I. The predicted size of each band is shown on the right side of the photograph. (c) The extent of the expression of galanin mRNA in the rat myometrium during pregnancy as determined by a densitometry. Photographs of PCR product bands, as shown in (a), were scanned and the extent of each mRNA expression was expressed based on the density ratio of the galanin bands in comparison to the β-actin bands. All data are expressed as the means$\pm$s.e.m ($n=3$). $^{**}P<0.01$ as compared with non-pregnant animals.

Discussion

The major findings of the present study are as follows: Galanin induces phasic contraction with or without oscillation in the rat myometrium by increasing $[Ca^{2+}]_i$ as well as by increasing Ca^{2+} sensitivity of the contractile apparatus. Galanin-induced increases in $[Ca^{2+}]_i$ are caused by both intracellular Ca^{2+} release and Ca^{2+} influx from extracellular space. The responsiveness of the rat myometrium to galanin does not change during pregnancy. The galanin mRNA is expressed in the rat myometrium and it is upregulated during mid-pregnancy. Rat myometrium expresses GALR2 but not GALR1 mRNA.

Galanin rapidly and transiently increased $[Ca^{2+}]_i$ and tension of the pregnant rat myometrium in the normal PSS (Figure 3a). Forty percent of the day 20 and day 22 pregnant strips examined here showed synchronized oscillatory movement of $[Ca^{2+}]_i$ and tension (Figure 1). The reason why oscillations were observed in only some of the strips is currently unknown. However, it was possible to speculate that variable responsiveness might be derived from the variation in the Ca^{2+} entry level in each tissue during resting state and/or during activation by galanin, presumably due to the variation of the membrane potential among the strips, because of the following reasons: (1) oscillations were completely abolished in the presence of diltiazem (2) occurrence of the oscillations increased in the presence of higher (2.5 mM) external Ca^{2+}. The mechanism responsible for the increase in the $[Ca^{2+}]_i$ in the initial transient phase and that in the oscillatory phase might be different. The former consisted of intracellular Ca^{2+} release (Figures 3c and 4a; discussed below), as well as the Ca^{2+} influx through both diltiazem-sensitive (Figure 3b) and diltiazem-insensitive Ca^{2+} channels, because a large part of the diltiazem-insensitive component was inhibited by Ni^{2+} (Figure 3c). On the other hand, the latter (oscillatory phase) was dependent on the L-type Ca^{2+} channel, since it was completely abolished by the pretreatment of the strips with diltiazem.

Under the condition in which Ca^{2+} entry was blocked by the presence of Ni^{2+} (Figure 3c) or by chelating the extracellular Ca^{2+} with EGTA (Figure 4a), galanin still induced small increases in $[Ca^{2+}]_i$ and tension. When the strip was exposed to a higher concentration of EGTA (0.5 mM), galanin-induced $[Ca^{2+}]_i$ elevation was completely abolished (Figure 4b), while the oxytocin-induced one remained (Figure 4c). Because oxytocin has been shown to activate PLC through a G protein and generate inositol 1,4,5-trisphosphate (IP_3),

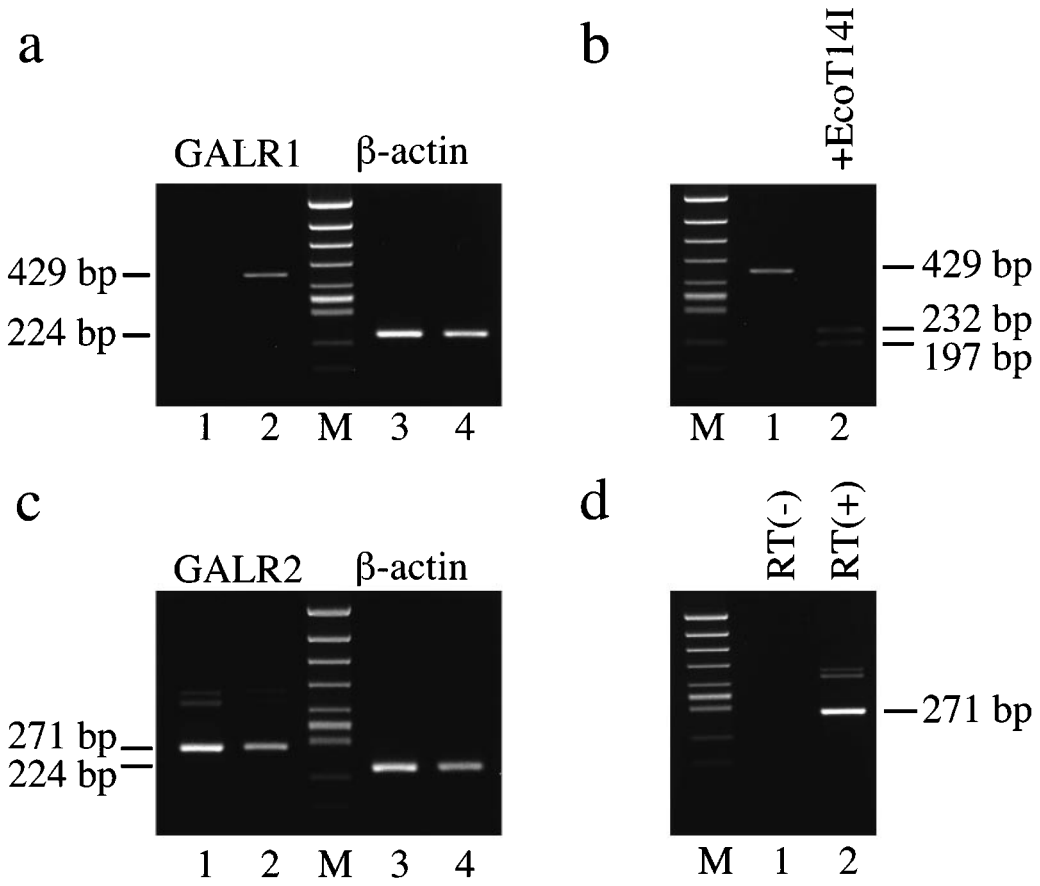

Figure 7 Detection of GALR1 and GALR2 mRNA by RT-PCR in the rat myometrium. (a) Detection of GALR1 mRNA by RT-PCR in the rat myometrium and ileum. PCR amplifications for GALR1 was performed for 35 cycles using total RNA prepared from myometrium (Lane 1) and ileum (Lane 2). The predicted size of PCR product is 429 bp, which is shown on the left side of the photograph. RT-PCR was performed for β-actin as an inner control (Lane 3; myometrium, Lane 4; ileum). (b) Identification of the PCR product for GALR1 by digestion with the restriction enzymes. Lane 1, the PCR product for rat GALR1 without digestion. Lane 2, the PCR product for rat GALR1 as digested by *Eco*T14I. The predicted size of each band is shown on the right side of the photograph. (c) Detection of GALR2 mRNA by RT-PCR in the rat myometrium and ileum. PCR amplifications for GALR2 was performed for 35 cycles using total RNA prepared from myometrium (Lane 1) and ileum (Lane 2). The predicted size of PCR product is 271 bp, which is shown on the left side of the photograph. Lanes 3 (myometrium) and 4 (ileum) are bands for β-actin as an inner control (d) Lane 1, the PCR product for rat GALR2 without RT. Lane 2, the PCR product for rat GALR2 with RT. The predicted size of band is shown on the right of the photograph. In all panels, lane M represents the DNA size marker (M; $\phi \times 174$/*Hinc*II digest). The size of each band was, from top to bottom, 495, 392, 345 + 341 + 335, 297 + 291, 210 and 162 bp.

which causes Ca^{2+} release from the sarcoplasmic reticulum (Wray, 1993). Thus the mechanism of the oxytocin-induced contraction in the Ca^{2+}-free PSS with 0.5 mM EGTA depends on the Ca^{2+} release from the IP_3-sensitive store. The reason of the smaller $[Ca^{2+}]_i$ elevation in the Ca^{2+}-free PSS with 0.1 mM EGTA, and failure to induce the Ca^{2+}-release in the presence of higher concentration of EGTA by galanin may be due to smaller amount of production of IP_3 as a result of weak activation of G protein or PLC. These data indicated that Ca^{2+} release mechanism from the intracellular Ca^{2+} store may be partly responsible for the initial elevation of $[Ca^{2+}]_i$.

Our next question was whether or not galanin modulates the Ca^{2+} sensitivity of the myometrium. To resolve this, we utilized myometrial strips permeabilized by β-escin, which enabled us to clamp the $[Ca^{2+}]_i$ without disrupting receptor/signal transduction pathways and contractile machinery (Kobayashi *et al*., 1989). Galanin caused an enhancement of Ca^{2+}-induced contraction ($[Ca^{2+}]_i = 0.3$ μM) in the presence of GTP though the enhancement was much smaller than that induced by GTPγS (Figure 5a). The presence of 1 mM GDPβS, which inhibits G-proteins, completely abolished the Ca^{2+}-sensitizing effect by galanin, as well as that by GTPγS (Figure 5b). A possibility that this effect might be mediated by the Ca^{2+} release from the internal store could be ruled out because the internal store was depleted by a Ca^{2+} ionophore, ionomycin and because the increase of the Ca^{2+} sensitivity by galanin was not transient but sustained. These results indicated that galanin activates G-protein, and thus, increases the Ca^{2+} sensitivity of the contractile apparatus of the rat myometrium.

Various contractile hormones and neurotransmitters bind their specific receptors and activate G-proteins coupled to different ion channels and enzymes including PLC. PLC metabolizes phosphatidylinositol and produces IP_3 and diacylglycerol. The former causes Ca^{2+} release from internal Ca^{2+} store and the latter activates protein kinase C. Many PLC-coupled receptor agonists have been shown to increase Ca^{2+} sensitivity of the contractile apparatus of smooth muscle. In the rat myometrium, Ca^{2+}-sensitizing mechanism is also reported to be involved in the contraction induced by contractile agents, such as oxytocin, carbachol, prostaglandin F2α and prostaglandin E_2 (Izumi *et al*., 1995, 1996). Thus, the

present results demonstrating that galanin induces Ca^{2+} influx, intracellular Ca^{2+} release and increased Ca^{2+} sensitivity indicate that galanin is one of the PLC coupled contractile agents in the myometrium.

Although galanin has been found to be involved in the regulation of avian oviposition, its involvement in the parturition of mammalian species has not yet been assessed. Among several hormones involved in parturition, oxytocin has been proposed as a key regulator of parturition (Soloff *et al*., 1979). Uterine sensitivity to oxytocin increases markedly at term. If galanin contribute to the onset and/or maintenance of labor at term through such effects as its oviposition-inducing action in the avian oviduct, an increase in the responsiveness of the myometrium to galanin could be expected. We assessed the galanin-induced contraction of the myometrium from the rats of various gestational stages and compared its EC_{50} values and maximal responses (Table 1). Our data showed that there is no significant difference in either EC_{50} values or maximal responses among the non-pregnant, early- (day 4), mid- (day 11), late- (day 20) and term- (day 22) pregnant rat myometrium (Table 1), which indicated that uterine sensitivity to galanin does not change throughout pregnancy.

We considered that another possible involvement of galanin in the parturition might be the up-regulation of the expression of galanin in the myometrium during pregnancy. For example, it has been demonstrated that the rat uterus itself is the major site of oxytocin gene expression during the later stage of pregnancy, suggesting that oxytocin acts as a paracrine or autocrine mediator (Lefebvre *et al*., 1992). Immunohistochemically, galanin-like immunoreactivity has been shown to exist in the myometrial nerves throughout the rat uterine horns and cervix, which suggests that galanin regulates myometrial tone as a neurotransmitter. In ovariectomized rats, exogenous application of 17β-estradiol caused a transient increase in galanin mRNA expression in uterus as well as in the pituitary (Vrontakis *et al*., 1993). However, the same report indicated that galanin mRNA was detected only in the endometrial stromal cells but not in the estrogen-treated myometrium by use of *in situ* hybridization. This result does not agree with the results showing galanin-like immunoreactivity in the myometrial nerves (Lakomy *et al*., 1995; Shew *et al*., 1992). We supposed that this discrepancy was due to the low sensitivity of the *in situ* hybridization method to detect the mRNA expression. Thus we performed RT-PCR, which is a more sensitive detector of the mRNA expression than *in situ* hybridization, and determined the change in the galanin mRNA expression in the rat myometrium during pregnancy. Our data showed that in the non-pregnant myometrium, the level of galanin mRNA expression was low, but increased to reach a maximal level on day 11. The galanin mRNA then decreased to that seen in the non-pregnant myometrium. (Figure 6a and c). Since estrogen is a dominant ganadotropin during pregnancy, the increase in the galanin mRNA expression may be mediated by estrogen. It has been reported that galanin mRNA could be induced by estrogen in the uterus as well as in the pituitary (Vrontakis *et al*., 1993). Recently, glucocorticoids such as dexamethasone and corticosterone have been shown to raise the galanin mRNA expression in the rat uterus (Vrontakis *et al*., 1996). Thus, corticosterone may be a candidate to modulate the galanin mRNA level in the uterus because its production increases during pregnancy (Waddell & Atkinson, 1994). These data support the idea that galanin-induced uterine contraction is regulated by the change in the galanin mRNA expression. Low level of expression at term seems to indicate that galanin does not participate in the onset or maintenance of labor. An increase in the galanin mRNA on day 4 might reflect the contribution of the galanin-induced longitudinal smooth muscle contraction to sperm transport. The physiological role of the high expression level of galanin mRNA in mid pregnancy, when the myometrium is quieted for the maintenance of pregnancy, is unknown. There could be another effect of galanin on myometrium other than regulating myometrial tone. We are tempted to speculate that galanin might be involved in the proliferation and/or hypertrophy of the uterine smooth muscle cells.

The functional experiments indicated that rat myometrial smooth muscle cells have galanin receptors, because galanin induced contraction of myometrium. Recently, two subtypes of galanin receptor have been cloned and termed GALR1 and GALR2, respectively (Habert-Ortoli, *et al*., 1994; Burgevin, *et al*., 1995; Parker, *et al*., 1995; Howard, *et al*., 1997). GALR1 mRNA is known to be expressed in hypothalamus, hippocampus, spinal cord and ileum in rat (Parker *et al*., 1995). Its expression in myometrial smooth muscle is not known. GALR2 mRNA was very recently cloned from rat brain and shown to be predominantly expressed in uterus and RINm5F insulinoma cell lines (Howard *et al*., 1997). It is also expressed in some smooth muscle tissues such as vas deferens, stomach and large intestine. Since the whole tissue of the uterus was used to prepare poly(A^+) mRNA, it is not clear whether or not GALR2 is expressed in the myometrium. Thus, we determined the mRNA expression of the two subtypes of galanin receptor and compared them with their expressions in ileum, which is known to express both types of galanin receptors. RT-PCR experiments indicated that only GALR2 mRNA was expressed in the rat longitudinal myometrium whereas both types were expressed in ileum (Figure 7). Thus, it is concluded that galanin-induced contraction of the rat myometrium may not be mediated by GALR1. GALR2 is the strongest candidate for the receptor involved in galanin-induced contraction of the rat myometrium. Recently, however, studies with guinea-pig gastric smooth muscle and galanin fragments suggested that there are more than three subtypes of galanin receptor (Gu *et al*., 1995). It is thus possible that other uncloned galanin receptors might be expressed in the myometrium and be responsible for the galanin-induced contractile effect.

In conclusion, our present study indicated that galanin induces rat myometrial contraction by increasing $[Ca^{2+}]_i$ and Ca^{2+} sensitivity of the contractile apparatus. Mechanisms to increase $[Ca^{2+}]_i$ involve Ca^{2+} influx and the Ca^{2+} release mechanism from the intracellular store. Increase of the Ca^{2+} sensitivity is mediated by G-protein. RT-PCR experiments revealed that rat myometrium possesses GALR2 but not GALR1. Galanin-induced myometrial contraction was found to be regulated, at least in part, by the change of galanin mRNA expression in the myometrium, but not by the change of the myometrial sensitivity to galanin, indicating that galanin might be a paracrine or autocrine mediator in the rat myometrium.

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