Pregnant rat myometrial cells show heterogeneous ryanodine- and caffeine-sensitive calcium stores

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Pregnant rat myometrial cells show heterogeneous ryanodine- and caffeine-sensitive calcium stores. Am. J. Physiol. 277 (Cell Physiol. 46): C243–C252, 1999.—Intracellular Ca2+ release channels such as ryanodine receptors play crucial roles in the Ca2+-mediated signaling that triggers excitation-contraction coupling in muscles. Although the existence and the role of these channels are well characterized in skeletal and cardiac muscles, their existence in smooth muscles, and more particularly in the myometrium, is very controversial. We have now clearly demonstrated the expression of ryanodine receptor Ca2+ release channels in rat myometrial smooth muscle, and for the first time, intracellular Ca2+ concentration experiments with indo 1 on single myometrial cells have revealed the existence of a functional ryanodine- and caffeine-sensitive Ca2+ release mechanism in 30% of rat myometrial cells. RT-PCR and RNase protection assay on whole myometrial smooth muscle demonstrate the existence of all three ryr mRNAs in the myometrium: ryr3 mRNA is the predominant subtype, with much lower levels of expression for ryr1 and ryr2 mRNAs, suggesting that the ryanodine Ca2+ release mechanism in rat myometrium is largely encoded by ryr3. Moreover, using intracellular Ca2+ concentration measurements and RNase protection assays, we have demonstrated that the expression, the percentage of cells responding to ryanodine, and the function of these channels are not modified during pregnancy.

In this connection, it is well known that during pregnancy the uterus undergoes dramatic modifications of its form and function and displays remarkable and essential changes in its electrical, mechanical, and biochemical characteristics (2, 17, 21). For example, 1) the density of Na+ channels and the number of gap junctions increase during pregnancy (16, 29), and 2) mRNAs encoding voltage-dependent Ca2+ channel (VDCC) subunits also increase in the pregnant rat myometrium (44). Although little is known about possible gestation-dependent expression and the role of RyRs, recent studies suggest that CICR may play a role in the EC coupling in rat myometrium at the end of gestation (39, 42).

Two major classes of Ca2+ channel family are involved in the release of Ca2+ from intracellular stores: the inositol trisphosphate (InsP3)-gated Ca2+ release channel [InsP3 receptors (InsP3Rs) and RyRs (19, 41)]. Both comprise three main subtypes: InsP3RI-III and RyR1–3, respectively. RT-PCR experiments have revealed the expression of all three InsP3R subtypes in the myometrium during human pregnancy (31). Moreover, induction of Ca2+ release occurs with direct application of InsP3 to permeabilized myometrial cells or purified myometrial SR (4, 31). In addition, application of InsP3 to permeabilized myometrial cell preparations induces a contractile response (18, 38), suggesting a functional role for InsP3R during EC coupling in the myometrium. However, although InsP3Rs play an important role in Ca2+ homeostasis in the myometrium, their expression does not change during pregnancy (31). In contrast to InsP3Rs, little is known regarding the expression and function of ryanodine-sensitive Ca2+ release channels in the myometrium. Although RT-PCR experiments have revealed the presence of RyRs in human myometrium (24) and we have presented electrophysiological evidence for a ryanodine-sensitive cation channel in nonpregnant myometrium from rat (26), ryanodine fails to produce force or intracellular Ca2+ concentration ([Ca2+]i) transients in cultured myometrial cells from pregnant rats (1). Moreover, caffeine, generally accepted to be a universal activator of CICR, is unable to release Ca2+ from internal stores or to evoke contractions or [Ca2+]i transients in human and rat myometrium (1, 15, 35, 37). Taken together, these data are difficult to reconcile. One of the reasons for the disparities in the cited studies may be differences in experimental conditions, e.g., human vs. animal tissue,

**Ryanodine receptors (RyRs)** located in the sarcoplasmic reticulum (SR) membrane of muscle tissues have a predominant role in excitation-contraction (EC) coupling (40). In heart and some smooth muscles, RyRs are the molecular basis for the Ca2+-induced Ca2+ release (CICR) mechanism that contributes to mechanical activity (3, 40). However, in uterine smooth muscle (myometrium), where spontaneous contractile activity is dependent on spontaneous discharge of Ca2+ action potentials (45), the existence and role of CICR and, consequently, the involvement of the SR in spontaneous contractions are still a matter of controversy.

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cultured vs. fresh cells, and nonpregnant vs. pregnant myometrium.

We have used a combination of molecular biology [RT-PCR and RNAase protection assays (RPAs)] and measurement of [Ca$^{2+}$] (fluorophore dye indo 1 system) to examine freshly isolated rat myometrial cells to 1) identify which ryr genes are expressed in myometrial smooth muscle, 2) determine whether their mRNA transcripts are differentially expressed over the course of pregnancy, and 3) characterize RYR function during pregnancy.

**MATERIALS AND METHODS**

Collection of tissues. Experiments were performed on uteri from Sprague-Dawley and Wistar female rats. Animals were anesthetized with ether and killed by cervical dislocation.

Preparation of isolated myometrial cells. Uteri were re-moved and placed in a physiological salt solution (PSS) composed of (in mM) 130 NaCl, 5.6 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 11 glucose, and 10 HEPES, with pH adjusted to 7.4 with NaOH. The uterine horn was opened longitudinally, and under binocular control the endometrium and the circular muscle layer were removed. The remaining longitudinal muscle layer was cut into several pieces (1 x 1 mm), incubated for 10 min in Ca$^{2+}$- and Mg$^{2+}$-free PSS, and then incubated in Ca$^{2+}$- and Mg$^{2+}$-free PSS containing 0.09% (wt/vol) collagenase (type CLS1, Worthington), 0.045% pronase (type E, Sigma Chemical) were applied trial cells, as previously described (13). Agonists (ACh, caffeine, ryanodine, and oxytocin; Sigma Chemical) were applied to the recorded cell by pressure ejection from a glass pipette.

Measurement of [Ca$^{2+}$]$_{i}$. We used the Ca$^{2+}$-sensitive fluorophore indo 1 to assess dynamic changes in [Ca$^{2+}$]$_{i}$ in individual myometrial cells. Cells were loaded with 1 µM indo 1-AM (Calbiochem) for 30 min at room temperature (20 ± 0.5°C). Coverslips containing indo 1-loaded cells were then washed for 25 min with fresh PSS to remove extracellular indo 1-AM. [Ca$^{2+}$]$_{i}$ was estimated from the indo 1 fluorescence using indo 1-AFC (0.4% in PSS; 360 ± 10 nm) and dual emission (405 ± 10 and 480 ± 10 nm) (13). The recording apparatus included a Nikon (Diaphot 300) inverted microscope fitted with epifluorescence (×40 oil immersion objective). The intensities of transmitted light from a window slightly larger than the cell were simultaneously recorded by two photometers (model P100, Nikon), and single-photon currents were converted to voltage signals. Signals at each wavelength were digitized and stored on a personal computer by using a PC-Lab Card 812PG interface, with sampling at 17 Hz. The ratio (R = F$_{405}$/F$_{480}$, where F$_{405}$ and F$_{480}$ represent fluorescence at 405 and 480 nm) was calculated on-line and displayed with the two voltage signals on a monitor. [Ca$^{2+}$]$_{i}$ was estimated from the ratio of the fluorescence (12), with use of a specific calibration for indo 1 determined within myometrial cells, as previously described (13). Agonists (ACh, caffeine, ryanodine, and oxytocin; Sigma Chemical) were applied to the recorded cell by pressure ejection from a glass pipette for the period indicated on the records. No change in [Ca$^{2+}$]$_{i}$ was observed during control ejection of PSS. Each record of [Ca$^{2+}$]$_{i}$ response to agonists was obtained from a different cell, and each type of experiment was repeated for the number of cells (n) indicated. No differences were seen in the resting [Ca$^{2+}$]$_{i}$ value or in the agonist-induced [Ca$^{2+}$]$_{i}$ response between the two rat strains.

Tissue collection and RNA isolation. The following groups were compared: nonpregnant (random cycling) and timed-pregnant rats at 10, 16–17, 18–19, 20–21, and 22 days postcoitus (nonlabor), 22 days during parturition, and 1 or 3 days postpartum. Uterine horns were removed and opened along the line of placental attachment. Pups and placenta were removed, and the endometrium was carefully scraped. Animals were killed during parturition after delivery had initiated, and usually when at least two pups had been delivered. The myometrium was then washed to remove all traces of blood, cut in small fragments, directly frozen in liquid nitrogen, and stored at −70°C for later RNA preparation. Total RNA from skeletal muscle, uterus, and whole brain was isolated as described previously (7), resuspended in RNase-free H$_2$O, and stored at −70°C. All the samples had intact 18S and 28S RNAs, as judged by ethidium bromide staining after agarose gel electrophoresis. Rat brain, cerebellum, and skeletal muscle were handled in the same manner to provide positive control RNA for PCR and RPA experiments. No differences were seen in ryr mRNA expression between the two rat strains.

Oligonucleotide primers, RT, and PCR amplification. Non-degenerate sense and antisense primers (Oswell DNA Service, Southend-on-Sea, UK) were used. The mouse sequence, was used to amplify an RT reaction to 80 µl of buffer containing 50 mM KCl, 10 mM Tris·HCl (pH 9.0), 2.5 mM MgCl$_2$, 0.2 mM each dCTP, dGTP, dTTP, and dATP, 1 µl of RNA (Promega), and 0.1 nmol of 3' PCR primer. Reactions were incubated for 10 min at room temperature, then for 30 min at 42°C and 5 min at 95°C (to inactivate the reverse transcriptase). Subsequent PCR amplification of specific cDNAs was carried out by adding the entire RT reaction to 80 µl of buffer containing 50 mM KCl, 10 mM Tris·HCl (pH 9.0), 2.5 mM MgCl$_2$, 0.2 µm each dCTP, dGTP, dTTP, and dATP, 10 units of Taq polymerase (Promega). After a “hot start” (5 min at 95°C), samples were subjected to 30–35 cycles as follows: for ryr1 5 cycles of 45 s at 95°C, 2 min at 45°C, and 45 s at 72°C; then a further 30 cycles of 45 s at 95°C, 2 min at 50°C, and 45 s at 72°C; for ryr2 30 cycles of 30 s at 95°C, 1 min at 45°C, and 1 min at 72°C; for ryr3 30 cycles of 45 s at 95°C, 2 min at 50°C, and 45 s at 72°C; for InsP$_3$-RI 30 cycles of 30 s at 95°C, 2 min at 50°C, and 1 min at 72°C. In each case, a final
extension step was carried out at 72°C for 10 min. Amplified products were analyzed by electrophoresis in 1% (wt/vol) agarose gels. For the nested ryr2 PCR, 1 µl of the initial RT-PCR was used in the subsequent PCR with nested primers with an annealing temperature of 58°C.

Cloning and sequencing of RT-PCR products. PCR products were subcloned into pGEM-T or pGEM-T easy (Promega) by overnight ligation at 15°C in 30 mM Tris·HCl (pH 7.8), 10 mM MgCl₂, 10 mM dithiothreitol, 0.5 mM ATP, and 3 units of T4 DNA ligase. Subclones of each ryr PCR product were sequenced on both strands with use of a Sequenase II sequencing kit (Amersham).

RPA. RPAs were performed on three to six uterine samples for each date of pregnancy by use of the HybSpeed RPA kit (Ambion) according to the manufacturer’s instructions. Negative controls contained yeast RNA instead of rat RNA. Rat whole brain RNA was used as a positive control and was processed at the same time as the different uterine samples. Rat InsP₃RI and ryr1, ryr2, and ryr3 partial cDNA clones were linearized and transcribed in vitro using [α-³²P]UTP (800 Ci/mmol; Amersham). A rat actin cRNA (pTRI-b-Actin, Ambion) was also synthesized and used as an internal control for assay variability. After purification by passage over Nick columns (Pharmacia), cRNA probes (9×10⁵ cpm) were hybridized to total myometrial (50 µg) or whole brain RNA (25 µg) in hybridization buffer (Ambion) for 10 min at 68°C. Free cRNA (nonhybridized) was removed by digestion with a 1:25 dilution of a mixture of RNase A (1 mg/ml) and RNase T1 (20,000 U/ml) for 30 min at 37°C. Samples were ethanol precipitated and resuspended in loading buffer for separation on a 4% or 5% (wt/vol) polyacrylamide gel containing 8 M urea. The gels were dried and exposed to X-ray film (Dupont) at 270°C for up to 2 wk with use of intensifying screens. Quantitation was carried out by densitometry with use of a computer-driven image analysis system (Seescan). The ratio of InsP₃RI to β-actin or ryr to β-actin in brain was arbitrarily set to 1, and results from myometrial RNA were expressed relative to brain.

Statistics. Data were assessed by ANOVA followed by a post hoc test. Significance was set at P < 0.05. Values are means ± SE.

RESULTS

RyR Ca²⁺ release channels are present and functional in single myometrial cells at 16–17 days of pregnancy. Using drugs known to act on these channels, including ryanodine and caffeine, we first assessed the function of RyRs in myometrial cells. Microejection of 10 or 50 µM ryanodine near the cell for 30 s evoked a transient increase in [Ca²⁺]ᵢ, which rose to 135 ± 8 nM (n = 11) and 213 ± 20 nM (n = 43), respectively, from a resting value of 73 ± 4 nM (n = 220; Fig. 1A).

The ryanodine-induced [Ca²⁺]ᵢ response was not significantly modified in the absence of external Ca²⁺ (n = 15; not shown). Interestingly, however, ryanodine (10 and 50 µM) induced a [Ca²⁺]ᵢ increase only in 29% (n = 40) and 33% (n = 131) of tested cells, respectively. To verify that the absence of ryanodine response in the remaining cells was not due to a lack of cellular viability, we tested the effect of alternative agonists known to increase [Ca²⁺]ᵢ in myometrial cells via different mechanisms, i.e., KCl, which acts on VDCC, ACh and oxytocin, which act through production of InsP₃ subclasses. In contrast to ryanodine, all the cells responded to the application of KCl, ACh, or oxytocin. KCl (60 mM), ACh (10 µM), and oxytocin (1 µM) evoked a [Ca²⁺]ᵢ response in 100% (n = 65), 91% (n = 55), and 98% (n = 65) of tested cells, respectively. [Ca²⁺]ᵢ re-
responses were composed of a fast transient peak followed by a sustained small-amplitude phase (Fig. 1B). Preincubation of the cells with 10 µM verapamil, a potent inhibitor of VDCC, abolished the KCl-induced [Ca^{2+}] response (n = 15), whereas the fast transient peak of ACh- and oxytocin-induced [Ca^{2+}] response was unaffected (n = 10 for each agonist; not shown).

To further investigate the lack of RyR responses in most cells, we used an alternative agonist, caffeine. Caffeine (0.1–25 mM) induced a transient [Ca^{2+}] response in a concentration-dependent manner (Fig. 2A), with half-maximal response at 0.3 mM and maximal response at 5 mM (n = 50; Fig. 2B). In the absence of external Ca^{2+}, the [Ca^{2+}] response to caffeine (5 mM) was not altered (n = 10; not shown). As seen with ryanodine, only ~30% of tested cells exhibited a [Ca^{2+}] increase in response to each caffeine concentration used. Moreover, in 35% of tested cells, caffeine induced a decrease in [Ca^{2+}] (Fig. 2C). The maximal absolute value of this decrease was 17 ± 2 nM (n = 40), 19 ± 2.6 nM (n = 30), and 18 ± 2 nM (n = 50) for 0.1, 0.5, and 5 mM caffeine, respectively. To further investigate the mechanism of the caffeine-induced [Ca^{2+}] increase, we tested the effect of tetracaine, a potent blocker of the CICR mechanism (40).

In control experiments we observed that two successive caffeine-induced [Ca^{2+}] responses could be obtained in the same cell (n = 30), provided that a 15-min interval was allowed between the two applications of caffeine (Fig. 3A). When 500 µM tetracaine was added in the bath after the first caffeine-induced [Ca^{2+}] response, the second caffeine-induced [Ca^{2+}] response was blocked (n = 30; Fig. 3B).

Effect of pregnancy on ryanodine- and caffeine-induced [Ca^{2+}] response. We investigated whether RyR function changes during pregnancy by determining the effect of ryanodine and caffeine on [Ca^{2+}] in myometrial cells isolated at 16–17 and 20–21 days of pregnancy. In cells from the late-stage pregnancy (20–21 days), the resting value of [Ca^{2+}] was slightly increased: 102 ± 4.7 nM (n = 160) for 20–21 days compared with 72 ± 1 nM (n = 220) for 16–17 days (P < 0.05). However, neither the percentage of responding cells nor the amplitude of the [Ca^{2+}] responses induced by ryanodine (10–50 µM) or caffeine (0.5–5 mM) changed between the two stages of pregnancy (Fig. 4).
Isolation of partial cDNAs encoding rat RyRs. Partial cDNAs encoding rat RyRs were obtained from tissues known to express the different isoforms. Thus RT-PCR was carried out on total RNA from rat skeletal muscle RNA (rry1 and rry3) and whole brain RNA (rry2). For rry2, a nested PCR was performed. This procedure amplified 486-, 454-, and 445-bp fragments encoding rry1, rry2, and rry3 cDNA, respectively (results not shown). The PCR products were subcloned, and their identity was verified by DNA sequencing. The sequence of the rat skeletal muscle rry1 cDNA was 90 and 91% identical to the previously reported pig and rabbit nucleotide sequences, respectively (22). The rry2 cDNA from whole rat brain was 85 and 93% identical to human and mouse rry2 nucleotide sequences, respectively (27). The nucleotide sequence of the partial rry3 cDNA was 91% identical to the human rry3 nucleotide sequence and 100% identical to the mink RyR3 amino acid sequence (28).

Analysis of RyR channel expression by RT-PCR. The rry1-, rry2-, rry3-, and InsP3RI-specific PCR primers were used to screen cDNA reverse transcribed from total myometrial RNA isolated from nonpregnant, pregnant (10, 16, 19, and 21 days or during parturition), and postpartum rats (1 day postpartum). Although RT-PCR is not quantitative, the technique gave a clear indication of the presence of the corresponding rry mRNA in the sample tested (Fig. 5). Total RNA from whole brain (which expresses all the receptors (11, 27)) was used as a positive control for the PCR, and samples where cDNA was replaced by H2O were used to test for any contamination (results not shown). All three known rat rry mRNAs, as well as InsP3RI mRNA, were represented in each of the myometrial RNA samples, as well as in the sample from rat whole brain (Fig. 5). All PCR amplification products were as easily detectable on ethidium bromide-stained agarose gels as rat brain RT-PCR products, suggesting that these genes are well expressed in rat myometrium. Moreover, with 30 cycles of PCR, no important differences were detected between the different myometrial samples, suggesting that rry or InsP3RI mRNA expression does not change dramatically during pregnancy. Furthermore, little sample-to-sample variability was detected (Fig. 5). RT-PCR with specific primers designed to differentially amplify the short and the long form of rry3 (30) shows that the long form (expected to be caffeine sensitive) is expressed in the myometrium from pregnant rat (16, 19, 20, and 21 days and during delivery) and nonpregnant rat (results not shown).

Quantitative analysis of RyR channel expression by RPA. RNA probes complementary to rry1, rry2, rry3, and InsP3RI mRNA were each used in an RPA together with a rat β-actin cRNA to control for assay variability (Fig. 6). For quantitation of each rry subtype and InsP3RI, the level of mRNA was expressed as a ratio to β-actin mRNA and relative to the ratio of rry to β-actin or InsP3RI to β-actin mRNA seen in rat brain. Although the expression of γ- and α-actin changes during pregnancy, β-actin mRNA remains constant (6). The level of InsP3RI mRNA expression in rat myometrium was high (between 50 and 60% of InsP3RI/β-actin in rat brain; Fig. 6A). Of the three subtypes, rry3 has the highest level of mRNA expression in the myometrium (50–75% of rry3/β-actin in rat brain; Fig. 6C). rry2 mRNA was barely detectable in the myometrium, even after 2 wk of exposure, and was present at only 25% of the rry2/β-actin content of brain (Fig. 6B). In myometrial...
samples, ryr1 mRNA was below the level of detection of this technique. Expression of InsP$_3$R1, ryr2, and ryr3 mRNA remains constant from 10 days of pregnancy to 3 days after delivery and does not change from the nonpregnant level (Fig. 6).

**DISCUSSION**

The present work demonstrates that ryanodine-sensitive Ca$^{2+}$ release stores do exist in rat myometrial smooth muscle cells. Moreover, examining functional RyR protein with use of [Ca$^{2+}$]$_i$ measurements in single myometrial cells and expression of their corresponding mRNAs in whole myometrial smooth muscle, we observed that the expression and the function of these channels did not change during pregnancy.

Ryanodine- and caffeine-induced Ca$^{2+}$ release in single myometrial cells. Our [Ca$^{2+}$]$_i$ experiments have demonstrated the existence of a ryanodine- and caffeine-sensitive Ca$^{2+}$ release mechanism in the rat myometrium. This finding confirms previous reconstitution of a ryanodine-sensitive Ca$^{2+}$ release channel in that preparation (26). However, the present study is at variance with studies in which no effect of ryanodine or/and caffeine was seen on [Ca$^{2+}$]$_i$ in cultured rat myometrial cells (1). A possible reason for this is that,
in the study of Arnaudeau et al. (1), the myometrial cells had been cultured at least overnight and in some cases for several days before exposure to ryanodine and caffeine, and the expression of ryr mRNA may have been altered over the period in culture. It is well known that cell culture results in changes in phenotype, and it has been demonstrated in vascular smooth muscle that ryr mRNA and caffeine-sensitive \( \text{Ca}^{2+} \) responses are lost during the first (proliferative) stage of culture of smooth muscle (23, 36). Also, using an insufficient number of tested cells may give misleading results. Interestingly, we have observed ryanodine- and caffeine-induced \( \text{Ca}^{2+} \) responses in only ~30% of myometrial smooth muscle cells, as discussed below. The finding that only a subset of myometrial cells is sensitive to ryanodine and caffeine is of particular interest for the understanding of uterine pharmacology. This characteristic could explain why we and others have not observed contractile responses to caffeine in rat myometrium (18, 35). Caffeine has been shown to exert a potent inhibitory effect (relaxant action) on mechanical activity in a variety of smooth muscles, including myometrium (37). This relaxant action is due to a decrease in \( \text{Ca}^{2+} \), as observed in 35% of cells in the present study, and to a decrease in the \( \text{Ca}^{2+} \) sensitivity of the contractile apparatus (35, 37). The decrease in \( \text{Ca}^{2+} \) is mediated, at least in part, by the methylxanthine-related inhibitory effect of caffeine on cyclic nucleotide-dependent phosphodiesterases (9), resulting in the stimulation of \( \text{Ca}^{2+} \) extrusion and \( \text{Ca}^{2+} \) storage after activation of plasmalemmal and SR \( \text{Ca}^{2+} \) pumps (8, 20). It is thus probable that, in the whole uterine muscle,
the Ca\(^{2+}\) release effect of caffeine operating in a subset of cells is masked by the overall relaxant effect.

The large size of mRNAs encoding ryr isoforms (>16 kb) and the low level of expression make quantitation difficult (e.g., by Northern blotting). However, using RT-PCR and RPA, we have shown the presence of the three known ryr isoforms in rat myometrium during pregnancy and we have demonstrated that ryr3 mRNA is the major ryr mRNA expressed in myometrial tissues, whereas ryr1 is barely detectable. Using a cDNA probe corresponding to part of ryr3 cDNA, Hakamata et al. (14) reported expression of ryr3 mRNA in rabbit myometrium by Northern blotting. Furthermore, caffeine-insensitive Ca\(^{2+}\) release has been reported in rat myometrium (35), consistent with the presence of RyR3 in this tissue (mink RyR3 is also insensitive to caffeine). However, in recent experiments in which a full-length cDNA encoding the rabbit uterine RyR3 expressed in HEK-293 cells was used, the encoded Ca\(^{2+}\) release channel protein was sensitive to ryanodine and caffeine (5), suggesting that alternatively spliced mRNA variants (possibly with a premature termination codon) may be responsible for the different caffeine sensitivity of RyR3 in this tissue. However, our primers spanned the alternatively spliced exon; inasmuch as we only detected ryr3 mRNA, which included the alternatively spliced exon in the myometrium, this rules out the possibility that the alternatively spliced variant described by Miyatake et al. (30) is responsible for differential sensitivity to caffeine in the myometrium. Therefore, we can speculate that in rat myometrium the main Ca\(^{2+}\) release mechanism is dependent on RyR3, which can be activated by ryanodine and caffeine. However, although we did not detect any alternatively spliced mRNA variants with our RT-PCR, we cannot exclude their presence in other parts of this gene. ryr1 and ryr2 mRNAs are also expressed in the myometrium, but at a very low level, and we do not know whether these ryr mRNAs (if indeed they are translated) are coexpressed in the same cells as RyR3. As each Ca\(^{2+}\) release channel has a preferred mechanism of activation (e.g., Ca\(^{2+}\), voltage, or cyclic ADP-ribose) and can be activated by different stimuli, expression of more than one type of RyR in the myometrium could facilitate very precise regulation of [Ca\(^{2+}\)]\(_i\) in myometrial cells.

Effects of gestation on the myometrium. Pregnancy is associated with changes in uterine structure and function to accommodate the developing fetus and to prepare for parturition. The molecular basis for these changes is poorly understood but must certainly be linked to changes in gene expression. RT-PCR and differential display techniques have been used to identify mRNAs for which the level of expression changes during pregnancy (6). For example, the expression of gap junctions, Na\(^+\) channels, VDCC, and cytoskeletal and matrix proteins is sensible to the gestational state and seems to increase toward parturition (6, 16, 29, 44).

In our study the three ryr mRNA subtypes are expressed in all myometrial samples (nonpregnant, pregnant, delivery, and postpartum states), and microspectrofluorimetry has not revealed a gestational modulation of the ryanodine or caffeine [Ca\(^{2+}\)]\(_i\) response in rat myometrium. This absence of gestational control of the expression of Ca\(^{2+}\) channel release has also been seen for the three subtypes of the InsP\(_3\)R (31) and for the three RyRs in human myometrium (C. Martin, K. E. Chapman, S. Thornton, and R. H. Ashley, unpublished observations). Clearly, however, inasmuch as the whole uterus undergoes an enormous gain in size during pregnancy, the total expression of ryr mRNA in the uterus will increase to reflect the increased number of cells. However, the ratio of ryr to \(-\)actin did not change. The only change that we have detected in our preparations is a small increase of the [Ca\(^{2+}\)]\(_i\) from 72 to 101 nM between 16–17 days and 20–21 days of pregnancy. Using estrogen-treated rats to mimic a real pregnancy, Osa (33) also observed an elevated basal [Ca\(^{2+}\)]\(_i\) in isolated myometrial cells from estrogen-treated compared with nonpregnant rats. This [Ca\(^{2+}\)]\(_i\) change can be triggered by membrane depolarization occurring near term, which brings the resting potential of the myometrial cell close to the threshold of VDCC activation, or by the opening of receptor-operated channels under hormonal stimuli, allowing Ca\(^{2+}\) entry (33).
Importance of RyR3 in the myometrium during parturition. We have demonstrated that the myometrium contains caffeine-sensitive Ca\(^{2+}\) stores. However, as only 30% of the cells in rat myometrium are caffeine and ryanodine sensitive, the physiological function of these stores is unclear. In this regard, it is worth noting that transgenic mice homologous for a targeted disruption of the ryr3 gene show normal growth and reproduction (43). However, it is possible that caffeine-sensitive stores are involved in the amplification of other Ca\(^{2+}\) mechanisms, such as InsP\(_3\)-induced Ca\(^{2+}\) release or receptor-mediated Ca\(^{2+}\) influx. Contractions of the myometrium at the end of the pregnancy are important for the normal functions of the organ, and the cells containing RyR may play a pacemaker role by initiating the contractions needed for the delivery.

In conclusion, our demonstration of the existence of a functional ryanodine- and caffeine-sensitive Ca\(^{2+}\) release mechanism in 30% of rat myometrial cells is intriguing. It demonstrates that rat myometrial cells are not a homogeneous population and suggests that there are functional differences between the cells in terms of their contractile function. It raises important questions as to the main role of RyRs in the myometrium and, in the long term, may contribute to improvements in the clinical management of uterine dysfunction during pregnancy and childbirth.

This research was supported by the Wellcome Trust, Institut National de la Santé et de la Recherche Médicale Grant CR1 9806, and Conseil Régional d’Aquitaine Grant 9630117. J.-M. Hyvelin is a recipient of an Agence de l’Environnement et de la Maîtrise de l’Energie studentship.

The DNA sequences reported in this paper have been deposited in the GenBank/European Molecular Biology Laboratories database (accession nos. AF112256 and AF112257).

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Received 11 March 1999; accepted in final form 28 April 1999.

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