FEBS Letters 372 (1995) 6–12

FEBS 15996

Isolation and partial cloning of ryanodine-sensitive Ca^{2+} release channel protein isoforms from human myometrial smooth muscle

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Received 26 July 1995

Abstract Partial cDNAs of the ryanodine receptor were cloned using PCR analysis from reverse transcribed total and mRNA, extracted from freshly isolated pregnant, non-pregnant, and cultured human myometrial smooth muscle. The identity of these clones was confirmed by nucleotide sequencing of the fragments and indicate the expression of both the skeletal and brain ryanodine receptor isoforms in these preparations. In freshly isolated non-pregnant myometrial tissue, membrane fractions displaying specific [^{3}H]ryanodine binding activities were isolated using density gradient centrifugation. SDS-PAGE of the sucrose gradient fractions indicated the specific comigration of a polypeptide with a molecular mass of ~544 kDa with the ryanodine binding activity.

Key words: Ryanodine; Myometrium; Human; Smooth muscle

1. Introduction

The uterus undergoes regular spontaneous activity both in vivo and in vitro and this activity may be modulated by agonists such as oxytocin and prostaglandins [1]. These substances bind to specific cell membrane receptors which initiate a series of biochemical and voltage-regulated pathways that lead to the production of transient elevations in cytoplasmic free Ca^{2+}, from non-mitochondrial intracellular stores and influx from the extracellular environment [2]. In human myometrium, both oxytocin and vasopressin are known to generate the second messenger, inositol 1,4,5-trisphosphate ($InsP_3$) [3], which regulates the release of intracellular Ca^{2+} from an $InsP_3$-sensitive store. This involves a specific $InsP_3$ receptor, which modulates many processes, such as excitation-contraction coupling and the influx of extracellular calcium through cell surface calcium channels [4]. A second mechanism capable of releasing intracellular Ca^{2+} is known to exist in skeletal, cardiac and smooth muscle which is sensitive to ryanodine and Ca^{2+} [4,5,6].

Ryanodine has been demonstrated to bind specifically and at nanomolar concentrations to the skeletal and cardiac ryanodine receptor Ca^{2+} release channels (RyR) on the sarcoplasmic reticulum (SR). At nanomolar concentrations, ryanodine locks the channel into a permanently open subconductance state, while at concentrations above 10 μM, ryanodine completely closes the channel [7,6]. Previous work on cultured human myometrial cells has demonstrated that a ryanodine-sensitive Ca^{2+} release mechanism is operational [8] and preliminary studies indicate the expression and isolation of a ryanodine binding protein in both myometrial cultured cells and whole tissue [9].

Three structurally distinct forms of the ryanodine-sensitive Ca^{2+} release channel complex have been identified, RyR1, RyR2 and RyR3 [10,6]. The skeletal (RyR1) and cardiac (RyR2) channels comprise 30S homotetrameric complexes of ~565 kDa subunits that form a 'four leaf clover-like' structure and display a morphology identical to the 'feet' structures that span the T tubule/SR junctional gap [11]. RyR1 is found predominantly in skeletal muscle and has also been identified in oesophagus and regions throughout the brain [12]. In skeletal muscle it is probably directly associated with a dihydropyridine receptor protein [13] located in the T tubular membrane. The entire complex is thought to operate as a voltage sensor where depolarisation of the T-tubular membrane leads to the release of Ca^{2+} from the SR. The Ca^{2+} sensitivity of the RyR1 system may enable positive feedback and the regenerative release of large quantities of Ca^{2+} from the SR [6]. The RyR2 channel found in cardiac muscle appears to be entirely dependent on cytoplasmic Ca^{2+} derived as a result of Ca^{2+} influx from the external environment [14]. Positive feedback of Ca^{2+} on RyR2 leads to the rapid release of Ca^{2+} from the cardiac SR. Both RyR1 and RyR2 demonstrate similar conductance and pharmacological properties as well as 66% identical amino acid sequence and similar membrane topology [15]. Functionally, RyR1 and RyR2 are potentiated by Ca^{2+}, ATP and inhibited by Mg^{2+} and ruthenium red [6,16]. There are also putative binding sites for calmodulin on both the skeletal and cardiac ryanodine receptors, located in distinct modulator binding regions within the protein, which may play an important role in the regulation of the Ca^{2+} release channel [17]. The skeletal and cardiac ryanodine systems are also sensitive to activation by caffeine with the cardiac ryanodine channel being most sensitive [18].

A third isoform of the ryanodine receptor/channel complex (RyR3) has been identified in rabbit brain and also appears to be expressed in mink lung epithelial cells after treatment with TGF-β [19,20]. This isoform appears to be expressed in many cell types including rabbit vascular smooth muscle cells [12,20]. At present the human RyR3 isoform has only been identified and partially sequenced in an established cell line [21]. The amino acid sequence of the RyR3 isoform, deduced from the

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Abbreviations: RyR, ryanodine receptor; RyR1, skeletal ryanodine receptor; RyR2, cardiac ryanodine receptor; RyR3, brain ryanodine receptor; SR, sarcoplasmic reticulum; $InsP_3$, inositol 1,4,5-trisphosphate.

SSDI 0014-5793(95)00924-8

nucleotide sequence of the mink lung and rabbit brain cDNA, indicates an overall identity of 70% with both RyR1 and RyR2.

However, there are few reports which describe the biochemical and molecular characteristics of the RyR3. Ryanodine binding proteins have been isolated from canine and porcine aortic smooth muscle where the Ca^{2+} release channel was shown to have similar properties to the skeletal and cardiac ryanodine receptor channel complexes [22]. Similarly, in toad visceral smooth muscle, the purified RyR channel migrated with a similar mobility to the cardiac ryanodine receptor on SDS-PAGE and cross-reacted with a monoclonal antibody to the canine cardiac ryanodine receptor [23]. It has also been reported that the RyR3 system in mink lung is not activated by caffeine [19].

This paper describes the expression of two isoforms of the ryanodine receptor from human myometrium. Recently it has been demonstrated that two ryanodine receptor isoforms are expressed in each type of porcine striated muscle and all ryanodine receptor isoforms are expressed in a number of regions of the nervous system [12]. The possible functional significance of two isoforms expressed in human myometrial smooth muscle remains to be determined. Parts of this work have been published in abstract form [9,24].

2. Materials and methods

2.1. Tissue preparation and culture techniques

Myometrial tissue from the lower uterine segment was taken, with informed consent, from patients undergoing either hysterectomy (women under 40 years of age) or elective caesarean section. Ethical approval was obtained from Newcastle Area Health Authority. Myocytes were prepared using a dispase/collagenase digestion [25] and maintained in M199 (Gibco Ltd) supplemented with 10% foetal calf serum, 1% glutamine and 2% penicillin/streptomycin. Cells were grown to confluence in culture flasks before being passaged onto plastic multiwells for $^{45}Ca^{2+}$ efflux studies. Cells were not used beyond passage number 6. Fresh tissue for isolation of total RNA and mRNA was quickly taken and transported frozen in liquid nitrogen. Tissue required for the purification of the ryanodine receptor was washed and transported, on ice, in 0.3 M sucrose containing protease inhibitors before being snap-frozen in liquid nitrogen and stored at −90°C, unless processed immediately.

2.2. $^{45}Ca^{2+}$ efflux from saponin-permeabilised cultured cells

Confluent cells, grown in 12 well multiwells, were washed with a balanced salt solution containing (mM): 135 NaCl, 5.9 KCl, 1.5 $CaCl_2$, 1.2 $MgCl_2$, 11.6 HEPES and 11.5 glucose, pH 7.3 to remove the culture medium before being fixed to a mechanical shaker. Cells were permeabilised with 15 μg/ml saponin for 15 min in the following skinning solution (mM): 120 KCl, 10 HEPES, 2 $MgCl_2$, 1 ATP and 1 EGTA, pH 7.0. The efficiency of skinning was routinely checked using Trypan blue exclusion staining. Traces of saponin were removed by washing the cells with a loading buffer containing (mM): 120 KCl, 10 HEPES, 5 $MgCl_2$, 5 ATP, 0.44 EGTA, 5 NaN_3 and 0.12 $CaCl_2$, pH 6.89. The mitochondrial Ca^{2+}/ATPase inhibitor NaN_3 was used to eliminate any contribution of Ca^{2+} released from mitochondrial pools to the overall efflux of Ca^{2+}. Non-mitochondrial intracellular stores were loaded with 10 μCi of $^{45}Ca^{2+}$ per well for 10 min in 0.5ml of the loading buffer. Loading was terminated by quickly washing three times in ice cold loading buffer prior to the start of efflux. The efflux buffer contained (mM): 120 KCl, 10 HEPES, 1 ATP, 3 EGTA and 5 NaN_3. The amount of $CaCl_2$ and $MgCl_2$ required to give solutions of known free Ca^{2+} concentration was calculated using the program REACT [26] and resting Ca^{2+} was taken to be 100 nM. $^{45}Ca^{2+}$ efflux was monitored at 2 min time intervals and the remaining $^{45}Ca^{2+}$ in the cells at the end of efflux was determined by solubilising the cells with 2% SDS. All experiments were carried out at room temperature (25°C) and the efflux of radiolabelled Ca^{2+} was determined by liquid scintillation counting. The loss of $^{45}Ca^{2+}$ at each 2 min time point was calculated from the cpm as a fraction of the total $^{45}Ca^{2+}$ remaining (including the final SDS fraction) within the cells at that time point.

2.3. cDNA synthesis and reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from both pregnant and non-pregnant human myometrial tissue by the guanidine isothiocyanate/lithium chloride method [27]. Prior to first strand cDNA synthesis, samples were treated with DNase I (FPLC pure, RNase free, Pharmacia) at 37°C for 30 min in 6 mM $MgCl_2$, 40 mM Tris-HCl, pH 7.5. Poly(A)$^+$ mRNA was prepared from cultured myometrial cells using a Quickprep mRNA purification kit (Pharmacia), following manufacturers instructions. The DNase I treated RNA (5μg or 0.5μg of Poly(A)$^+$ mRNA) served as the template for first strand cDNA synthesis using avian myeloblastosis virus reverse transcriptase (Pharmacia) or Superscript RT (Gibco BRL) using 200 U per μg RNA. Specific 3′ oligonucleotides were used as primers for first strand cDNA synthesis. A quarter of the reverse transcriptase reaction product was amplified, using standard PCR methods, in a 100 μl reaction volume using *Taq* polymerase (BRL Gibco). Amplification conditions were as follows: denaturing; 94°C for 5 min, primer annealing; 50°C for 2 min, extension; 72°C for 3 min, followed by 30 cycles of denaturing; 94°C for 1 min, primer annealing; 45°C for 2 min, extension; 72°C for 3 min and one final cycle, denaturing; 94°C for 1 min, primer annealing; 50°C for 2 min and extension; 72°C for 10 min. The amplification conditions for cultured cell cDNA primed with oligonucleotides designed to the RyR3 sequence were identical except that the primer annealing was carried out at 55°C.

2.4. Synthetic oligonucleotides

Oligonucleotides were designed from the human type-1 ryanodine receptor cDNA sequence [28] with incorporated restriction endonuclease sites for subsequent cloning of the PCR amplified products. The following oligonucleotides were used: RyR1/5′, AAGGTGCCCCTGGAATTCTTTGCGGGAG (sense) containing an *Eco*RI site and RyR1/3′, GTGTGTTCTGTCGGATCCTTGTTTATCAAA (antisense) containing a *Bam*HI site to amplify a product of 999 bp. The following oligonucleotides were designed from the reported type 3 human ryanodine receptor cDNA sequence [21]: RyR3/5′, TCACCTATTGGAATTCGCAATGGGCT (sense) containing an *Eco*RI site and RyR3/3′, AGTAGTTGGCTAAGCTTTGCTCTTGTAAT (antisense) containing a *Hin*dIII site to amplify a product of 477 bp. All oligonucleotides were obtained from the Molecular Biology Service Unit, University of Newcastle upon Tyne.

2.5. Nucleotide sequencing of the PCR derived type-1 and type-3 ryanodine receptor clones

The DNA sequences of the PCR derived clones were determined by the dideoxy chain termination method [29] after subcloning appropriately designed fragments into the vectors M13mp18 and M13mp19 [30]. Clones from different PCR mediated amplifications of the ryanodine receptor fragments were sequenced to verify that the amplified products were homogeneous and specific. The preparation of oligonucleotides and sequencing were performed as previously described [29,30,31].

2.6. Isolation of heavy SR vesicles from myometrial smooth muscle

Heavy SR vesicles were prepared from human myometrium obtained at hysterectomy using previously described methods [32]. The tissue was minced and homogenised in 7 vols. of ice-cold 0.1 M NaCl, pH 7.5, containing 10 mM Tris-HEPES, 0.5 mM EDTA and protease inhibitors (0.1 mM aprotinin, 1 μM leupeptin, 1 μM pepstatin, 1 mM benzamidine and 0.2 mM PMSF) at 4°C for 2 × 30 s periods in a Waring blender, followed by centrifugation at 2600 × *g* for 25 min at 4°C. The supernatant was then filtered through two layers of cheesecloth and centrifuged at 33000 × *g* for 30 min at 4°C. The resulting pellets were resuspended in extraction buffer (0.6 M KCl/10 mM KPipes, pH 7.0), containing 100 μM EGTA, 90 μM Ca^{2+}, 0.2 mM PMSF and 1 μM leupeptin, and left on ice for 60 min. The suspension was centrifuged at 35000 × *g* for 60 min at 4°C and the pellets were resuspended in extraction buffer and layered on the top of a 20–45% (w/w) linear sucrose gradient. The gradients were centrifuged at 26000 × *g* for 16 h at 2°C and the resulting gradients fractionated into 2 ml fractions. Membranes sedimenting at 25–35% sucrose were collected, diluted with 2 vols. of 0.4 M KCl, centrifuged at 30000 × *g* for 60 min at 2°C, resuspended in 0.3 M sucrose, 5 mM KPipes, pH 7.0 and stored at −80°C. Protein was

determined for each fraction by the method of Lowry et al. [33] using BSA as a standard.

2.7. [^{3}H]Ryanodine binding assay

Heavy SR vesicles containing the RyR were incubated at 12°C for approx. 24 h in 100 μl of reaction mixture containing 20 mM KPipes, pH 7.4, 0.6 M KCl, 50 μM EGTA, 200 μM Ca^{2+}, 6 mM AMP, 0.1 mM PMSF, 10 mM leupeptin and 12.5 nM [^{3}H]ryanodine. Bound and free [^{3}H]ryanodine were separated by a filter assay [34] with several modifications. Aliquots of 40 μl of the vesicle suspensions were diluted 25-fold with ice-cold water, placed on a Whatman GF/B glass fibre filter presoaked in 1% (w/v) polyethyleneimine, and rinsed three times with 5 ml of ice-cold 0.1 M KCl, 1 mM KPipes, pH 7.0. Total radioactivity and bound radioactivity as determined after filtration were measured by liquid scintillation counting. Non-specific binding, which was assumed to be linear with respect to ryanodine concentration, was determined in the presence of 1 mM unlabelled ryanodine.

2.8. SDS-PAGE

Samples were electrophoresed in 4–15% gradient gels, using the Pharmacia Phastsystem with the buffer system of Laemmli [35]. Samples were denatured at 90°C for 5 min in 2% SDS, 2.5% β-mercaptoethanol, 0.1 M Tris-HCl, pH 6.8 and 10% glycerol, and electrophoresed at 15°C. Gels were stained with 0.05% Coomassie brilliant blue R250 in 30% methanol and 10% acetic acid.

2.9. Materials

[$^{45}Ca^{2+}$]-, [^{35}S]- and [^{3}H]ryanodine were purchased from Amersham International, U.K. Medium 199 and other tissue culture materials were from Gibco Ltd, UK. Ryanodine and caffeine were obtained from Sigma Chemical Co., Poole, Dorset, UK. RNase-free DNase I, reverse transcriptase and Klenow fragment were from Pharmacia, Uppsala, Sweden and Superscript RT from Gibco BRL. All oligonucleotide probes were produced by the University Facility for Molecular Biology, University of Newcastle upon Tyne. *Taq* polymerase was from BCL, UK. All other chemicals used were of analytical grade or greater.

3. Results

3.1. $^{45}Ca^{2+}$ efflux from permeabilised cultured cells

3.1.1. Effects of ryanodine and caffeine. Microspectrofluorimetry of intact cultured myometrial cells with Fura-2 suggested that a ryanodine-sensitive, caffeine-insensitive Ca^{2+} release mechanism may be operational [8]. In order to directly demonstrate a caffeine-insensitive, ryanodine-sensitive Ca^{2+} release activity, the non-mitochondrial stores of permeabilised human myometrial cells were loaded with $^{45}Ca^{2+}$. Using this technique, the composition of the medium bathing the cells can be controlled accurately and experimental solutions may be directly exposed to the internal membranes. The effects of caffeine and ryanodine were examined in permeabilised cultured cells and Fig. 1A illustrates the effect of 10 mM caffeine on the basal efflux of $^{45}Ca^{2+}$. The results indicate that there is no caffeine-induced Ca^{2+} release in these cells. Fig. 1B demonstrates a typical response obtained from $^{45}Ca^{2+}$ loaded cells to a 2 min incubation with 1 μM ryanodine (■). Ryanodine increased the rate of $^{45}Ca^{2+}$ loss at 1 μM, confirming the presence of a ryanodine-sensitive Ca^{2+} release process. However, this ryanodine-sensitive Ca^{2+} release was augmented in the presence of 10 mM caffeine (●).

3.1.2. Isolation of a ryanodine binding protein from whole tissue. Experiments were performed to confirm the presence of a ryanodine receptor/channel in human myometrial cells. Freshly isolated non-pregnant tissue obtained from hysterectomy was used for membrane preparation since the large amount of tissue required could not be obtained easily from the culture system. A heavy membrane fraction was isolated and specific [^{3}H]ryanodine binding could be detected in the 25–35% sucrose region of the sucrose density gradient (Fig. 2A). Fig. 2B compares specific [^{3}H]ryanodine binding to the pooled fractions from the lower third of the gradient and a comparable SR membrane fraction isolated from rabbit skeletal muscle. The specific [^{3}H]ryanodine binding activity of myometrial mem-

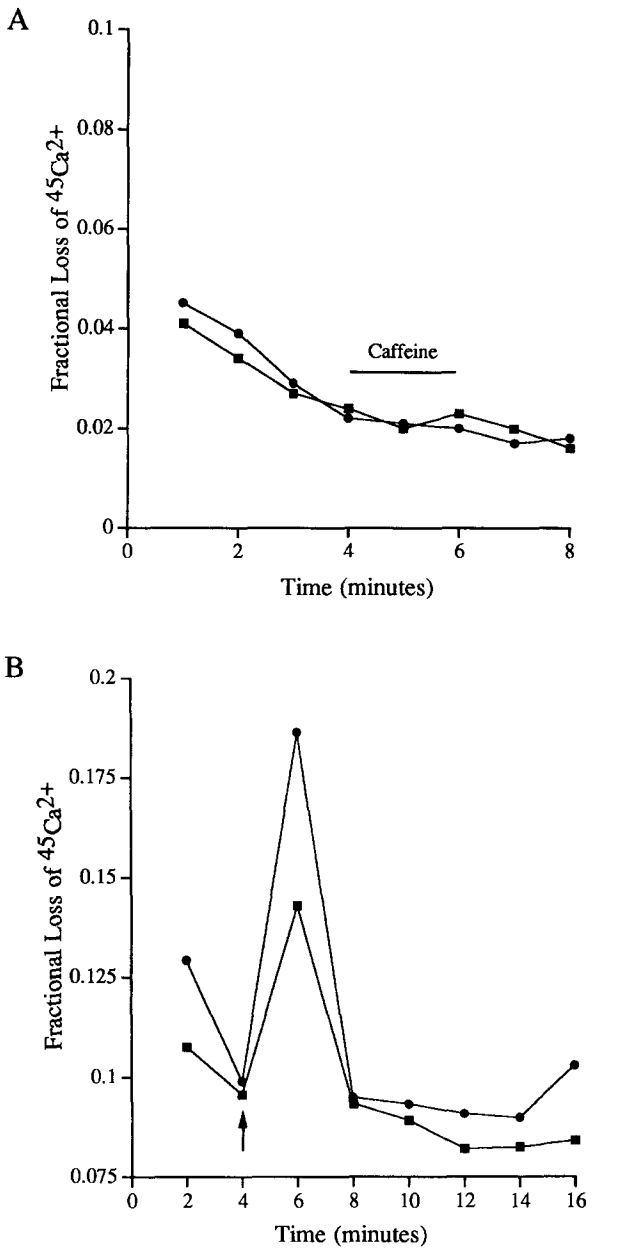

Fig. 1. Effect of ryanodine and caffeine on $^{45}Ca^{2+}$ efflux from permeabilised myometrial smooth muscle cells. (A) $^{45}Ca^{2+}$ efflux from saponin-permeabilised cells under control conditions (■) and after a 2 min application of 10 mM caffeine (●). (B) The effects of 10 mM caffeine on the response to 1 μM ryanodine in permeabilised cultured human myometrial smooth muscle cells. (■) shows a control response to a 2 min application of ryanodine (arrow) and (●) shows the augmentation of the 1 μM ryanodine-induced Ca^{2+} release in the presence of caffeine. Caffeine was applied throughout the time course of efflux. Experiments were carried out at room temperature. The data presented are typical of six paired experiments.

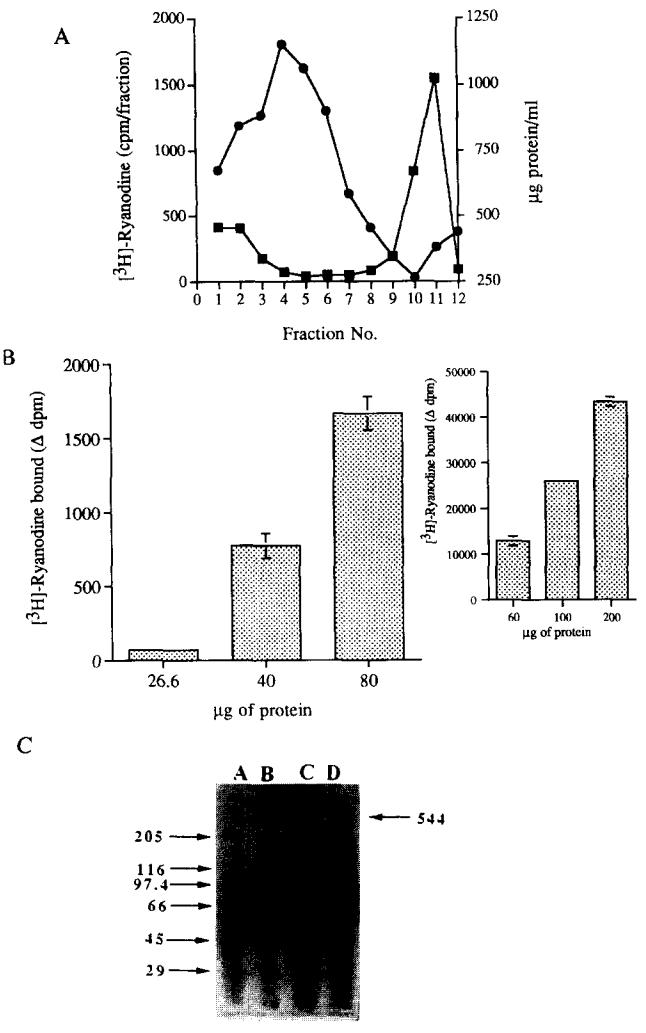

Fig. 2. Isolation and identification of a [^{3}H]ryanodine binding protein from myometrial membrane fractions. (A) [^{3}H]Ryanodine (■) and protein (●) sedimentation profile of myometrial heavy SR membranes centrifuged through a 20–45% linear sucrose gradient. (B) [^{3}H]Ryanodine binding to myometrial membrane fractions using 12.5 nM [^{3}H]ryanodine and varying protein concentration. The insert shows [^{3}H]ryanodine binding to skeletal muscle SR membranes using 12.5 nM [^{3}H]ryanodine. This clearly indicates the low levels of binding obtained with myometrial smooth muscle membranes. The data represent means ± S.E.M. for four independent experiments each with duplicate samples. (C) Coomassie blue-stained SDS-polyacrylamide gradient (4–15%) gel of human myometrial membranes isolated on sucrose density gradient, gradient fractions 5 (A and B) and pooled ryanodine binding fractions 10–12 (C and D). The arrow demonstrates a polypeptide band found only in the ryanodine binding peak fractions with an estimated molecular mass of ~544 kDa. The molecular mass standards shown are: carbonic anhydrase (29,000), egg albumin (45,000), bovine serum albumin (66,000), phosphorylase *b* (97,400), β-galactosidase (116,000) and myosin (205,000).

branes was approximately 10% of the levels found with skeletal muscle membranes (Fig. 2B). For all experiments non-specific binding was taken as the binding measured in the presence of 1mM unlabelled ryanodine in an identical sample and represented 25% of total binding. Fig. 2C shows a 4–15% SDS polyacrylamide gradient gel of the sucrose gradient fractions where a band with an apparent molecular mass of ~544 kDa comigrated with the [^{3}H]ryanodine binding peak. This band was absent in all other fractions examined.

3.2. *RT-PCR, cDNA cloning and cDNA sequencing of RyR1 and RyR3 specific clones*

To determine the expression of the human RyR isoforms in myometrial tissue, RT-PCR was employed. The isolated total RNA and mRNA was used for an initial reverse transcription step to produce cDNA, followed by amplification of specific sequences by the PCR method.

Using the published cDNA sequences for the human RyR1 and RyR3 receptors, specific oligonucleotide probes were designed for amplification of each isoform. The human RyR2 isoform sequence is not yet published and it was therefore not possible to design specific probes to investigate whether this isoform is also found in human myometrium. The specific PCR primers were designed to produce an 999 bp and 477 bp product, from the 3′ region of RyR1 and RyR3 cDNA sequences, respectively (Fig. 3). Although the sequences are related, the oligonucleotide primers were taken from regions that showed the greatest sequence diversity in order to maximise selectivity. The specific PCR primers were designed to produce a PCR product that encoded a section of the transmembrane spanning domains of each isoform. The purified RNA used for the initial reverse transcription reactions was treated with DNase to remove any contaminating genomic DNA so that only cDNA produced by the reverse transcription could serve as template to produce the correct size of PCR products.

cDNA sequence analysis of multiple independent isolates of the subcloned PCR products confirmed that each pair of primers was specific for one of the two ryanodine mRNA species. Sequence analysis of the cDNAs revealed an open reading frame of 837 bp for RyR1 and 456 bp for RyR3 and their deduced amino acid sequences are indicated (Fig. 4A,B). Controls which lacked the reverse transcriptase enzyme gave no detectable PCR product, which confirmed that both RyR1 and RyR3-specific PCR products were amplified from a cDNA template and not from genomic DNA.

Restriction enzyme analysis of the human RyR3-specific PCR products indicated that the *Hha*I restriction endonuclease site predicted from the human RyR3 nucleotide sequence [21], was not present in any of the samples. The absence of the *Hha*I site was confirmed by direct sequence analysis showing that the published T position was a C [21]. This change in the base pair alters the codon such that the amino acid arginine at this position is replaced with cysteine. The substitution to cysteine at this position is in agreement with the RyR1 amino acid sequence at position 4870 and the rabbit brain deduced amino acid sequence at position 4744 [28,20]. 837 bp of the RyR1 RT-PCR products were sequenced following their cloning into M13 and found to be identical to the published RyR1 sequence [28]. The RyR3-specific PCR primers amplified a 456 bp sequence which was 94% homologous to the rabbit brain RyR cDNA sequence at position 14061–14516 [20]. Sequence analysis of the two myometrial-specific ryanodine receptor isoforms revealed 79% identity in nucleotide sequence between the transmembrane spanning regions of these two isoforms.

4. Discussion

The present data describes the cloning and partial sequence analysis of cDNAs encoding two isoforms of the human ryanodine receptor channel (RyR1 and RyR3) in both pregnant and non-pregnant myometrial tissue and cultured myometrial

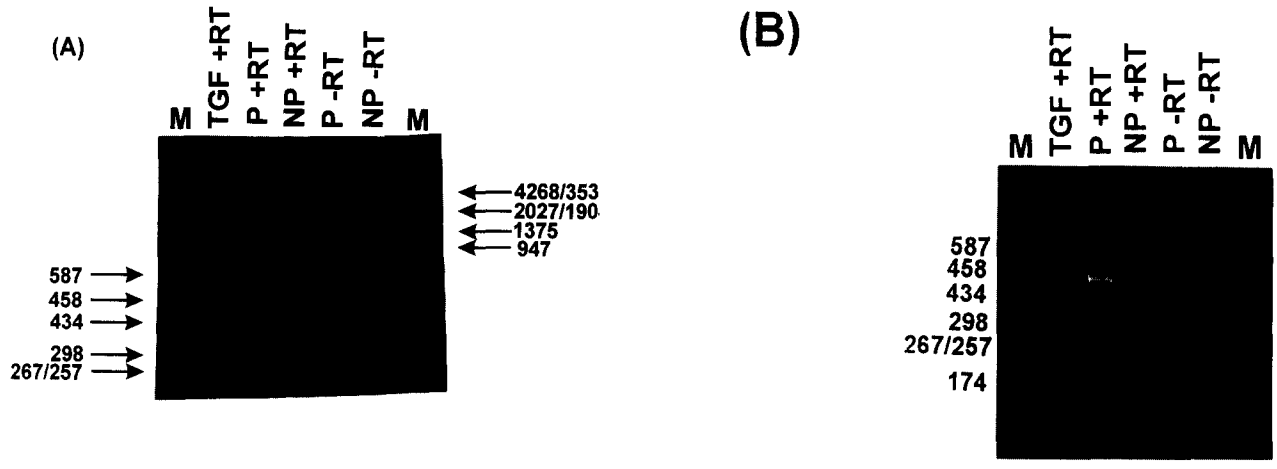

Fig. 3. RT-PCR of ryanodine receptors (RyR1 and RyR3) in human myometrial smooth muscle. Polyacrylamide gel (6%) electrophoresis of RyR1 and RyR3 RT-PCR products from human myometrial smooth muscle RNA. (A) The RyR1-specific oligonucleotides amplified a product of ~1 kb from cultured cells incubated with 1 ng/ml TGF-β for 24 h (TGF), pregnant tissue (P) and non-pregnant tissue (NP). (B) The RyR3-specific oligonucleotides amplified a product of ~500 bp from samples as indicated in A. Pregnant and non-pregnant RNA was treated with or without reverse transcriptase (± RT) to show that the RT-PCR products were amplified from cDNA only. The nucleic acid marker lanes are indicated (M).

smooth muscle cells. The work reported here also indicates that saponin-permeabilised myometrial smooth muscle cells are not activated by caffeine but exhibit ryanodine-sensitive Ca^{2+} release when the intracellular stores are loaded with $^{45}Ca^{2+}$. This ryanodine-sensitive Ca^{2+} release is augmented by the prior application of 10 mM caffeine to the permeabilised cell. This suggests that although caffeine does not release Ca^{2+} directly, it may modulate the activity of one or both isoforms in the presence of ryanodine by opening the CICR channel and allowing ryanodine to maintain them in an open state. This sensitisation of the ryanodine-induced Ca^{2+} response by caffeine may indicate the contribution of the RyR1 isoform which may be less abundant than the RyR3 isoform. The demonstration of a membrane fraction from myometrial tissue that specifically bound [^{3}H]ryanodine allowed the confirmation that a ryanodine-binding protein was indeed present in human myometrium.

In this study RT-PCR has been used successfully to demonstrate the expression of two isoforms of the ryanodine receptor in pregnant and non-pregnant human myometrial smooth muscle tissue. The clone corresponding to the RyR1 isoform was 837 bp in length and exhibits 100% identity to the reported human cDNA sequence [28], whereas the RyR3 specific clone was 456 bp in length and was identical to the reported human nucleotide sequence [21], except that it contained a single base pair substitution. The C for T change in the RyR3 clones at position 172 may represent an allelic variation and not a PCR artefact, as this substitution was present in all clones prepared from four independent samples. This was also confirmed by the absence of any digested fragments obtained by the restriction

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HMSM RyR1  GATGACGTGAAGGGGCAGTGGGACCGACTGGTGCTCAACACGCCGTCTTTCCCTAGCAAC 60
HMSM RyR3

HMSM RyR1  TACTGGGACAAGTTTGTCAAGCGCAAGGTCCTGGACAAACATGGGGACATCTACGGGCGG 120
HMSM RyR3

HMSM RyR1  GAGCGGATTGCTGAGCTACTGGGCATGGACCTGGCCACACTAGAGATCACAGCCCACAAT 180
HMSM RyR3

HMSM RyR1  GAGCGCAAGCCCAACCCGCCGCCAGGGCTGCTGACCTGGCTCATGTCCATCGATGTCAAG 240
HMSM RyR3

HMSM RyR1  TACCAGATCTGGAAGTTCGGGGTCATCTTCACAGACAACTCCTTCCTGTACCTGGGCTGG 300
HMSM RyR3

HMSM RyR1  TATATGGTGATGTCCCTCTTGGGACACTACAACAACTTCTTCTTTGCTGCCCATCTCCTG 360
HMSM RyR3

HMSM RyR1  GACATCGCCATGGGGGTCAAGACGCTGCGCACCATCCTGTCCTCTGTCACCCACAATGGG 420
HMSM RyR3            GGCTTCAAGACACTGAGGACCATTCTGTCATCTGTAACTCACAATGGC 48

HMSM RyR1  AAACAGCTGGTGATGACCGTGGGCCTTCTGGCGGTGGTCGTCTACCTGTACACCGTGGTG 480
HMSM RyR3  AAACAGTTGGTTCTGACTGTCGGTCTCCTGGCCGTGGTGGTTTATCTCTATACTGTGGTG 108

HMSM RyR1  GCCTTCAACTTCTTCCGCAAGTTCTACAACAAGAGCGAGGATGAGGATGAACCTGACATG 540
HMSM RyR3  GCTTTCAACTTCTTCCGCAAGTTCTACAACAAAAGCGAAGACGATGACGAGCCCGATATG 168

HMSM RyR1  AAGTGTGATGACATGATGACGTGTTACCTGTTTCACATGTACGTGGGTGTCCGGGCTGGC 600
HMSM RyR3  AAGTGCGACGACATGATGACGTGTTACCTTTTCCACATGTACGTGGGAGTGAGAGCAGGA 228

HMSM RyR1  GGAGGCATTGGGGACGAGATCGAGGACCCCGCGGGTGACGAATACGAGCTCTACAGGGTG 660
HMSM RyR3  GGTGGCATTGGTGATGAAATTGAAGACCCTGCTGGTGACCCTTATGAAATGTATCGCATT 288

HMSM RyR1  GTCTTCGACATCACCTTCTTCTTCTTCGTCATCGTCATCCTGTTGGCCATCATCCAGGGT 720
HMSM RyR3  GTCTTTGACATTACCTTTTTCTTCTTCGTCATTGTCATCTTGCTGGCCATCATTCAAGGT 348

HMSM RyR1  CTGATCATCGACGCTTTTGGTGAGCTCCGAGACCAACAAGAGCAAGTGAAGGAGGATATG 780
HMSM RyR3  CTTATTATTGATGCTTTCGGAGAGCTAAGAGACCAGCAGGAACAAGTACGAGAAGATATG 408

HMSM RyR1  GAGACCAAGTGCTTCATCTGTGGAATCGGCAGTGACTACTTTGATACGACACCGCAT    837
HMSM RyR3  GAGACTAAATGTTTCATCTGTGGGATTGGCAATGACTACTTTGACACA             456
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HMSM RyR1  D D V K G Q W D R L V L N T P S F P S N 20
HMSM RyR3

HMSM RyR1  Y W D K F V K R K V L D K H G D I Y G R 40
HMSM RyR3

HMSM RyR1  E R I A E L L G M D L A T L E I T A H N 60
HMSM RyR3

HMSM RyR1  E R K P N P P P G L L T W L M S I D V K 80
HMSM RyR3

HMSM RyR1  Y Q I W K F G V I F T D N S F L Y L G W 100
HMSM RyR3

HMSM RyR1  Y M V M S L L G H Y N N F F F A A H L L 120
HMSM RyR3

HMSM RyR1  D I A M G V K T L R T I L S S V T H N G 140
HMSM RyR3            G F K T L R T I L S S V T H N G 16

HMSM RyR1  K Q L V M T V G L L A V V V Y L Y T V V 160
HMSM RyR3  K Q L V L T V G L L A V V V Y L Y T V V 36

HMSM RyR1  A F N F F R K F Y N K S E D D E D E P D M 180
HMSM RyR3  A F N F F R K F Y N K S E D D D E P D M 56

HMSM RyR1  K C D D M M T C Y L F H M Y V G V R A G 200
HMSM RyR3  K C D D M M T C Y L F H M Y V G V R A G 76

HMSM RyR1  G G I G D E I E D P A G D E Y E L Y R V 220
HMSM RyR3  G G I G D E I E D P A G D P Y E M Y R I 96

HMSM RyR1  V F D I T F F F F V I V I L L A I I Q G 240
HMSM RyR3  V F D I T F F F F V I V I L L A I I Q G 116

HMSM RyR1  L I I D A F G E L R D Q Q E Q V K E D M 260
HMSM RyR3  L I I D A F G E L R D Q Q E Q V R E D M 136

HMSM RyR1  E T K C F I C G I G S D Y F D T T P H    279
HMSM RyR3  E T K C F I C G I G N D Y F D T          152
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Fig. 4. cDNA sequence analysis of RyR1 and RyR3-specific clones and deduced amino acid sequences from human myometrium. (A) Alignment of the nucleotide sequences of the human myometrial-specific RyR1 and RyR3 RT-PCR products. PCR products were cloned into M13mp18 and M13mp19 and sequenced as described in sectin 2. (B) Deduced amino acid sequences of the human myometrial RyR1 and RyR3 RT-PCR products. The myometrial RyR1 sequence is identical to residues 4691–4968 of the reported type1 ryanodine receptor cDNA sequence (Zorzato et al., 1990).

endonuclease *Hha*I. This restriction site has been predicted from the published RyR3 nucleotide sequence [21]. Analysis of the deduced amino acid sequence reveals that this single base pair difference alters the amino acid sequence from arginine to cysteine at this position and this site is identical to that found in the RyR1 isoform at residue 4870. We have been unable to detect the RyR2 isoform in myometrial smooth muscle as the human DNA sequence required to design specific primers for this isoform is at present unknown. The possible presence of this isoform in the myometrium remains to be determined before an accurate estimation of the contribution of the various isoforms in Ca^{2+} signalling can be established.

[^{3}H]Ryanodine binding assays using isolated heavy SR membranes from the myometrium confirmed the presence of a ryanodine binding activity in this tissue. The amount of bound [^{3}H]ryanodine was approximately 10% of that obtained with heavy SR membranes isolated from rabbit skeletal muscle, which contains a more extensive SR membrane system and so may reflect a difference in the contents of the receptor so leading to higher levels of specific [^{3}H]ryanodine binding. The comparatively reduced levels of [^{3}H]ryanodine binding protein isolated from the myometrium still enabled the estimation of its molecular mass by SDS gradient gel electrophoresis. Fractions isolated using sucrose density gradients were electrophoresed on a 4–15% gradient gel and a polypeptide of ~544 kDa was demonstrated in the fractions corresponding to the [^{3}H]ryanodine binding peak from the sedimentation profile, which was absent in all other fractions. The demonstration of the RyR using RT-PCR, while not allowing quantitative assessment of the isoforms present, would appear to agree with the [^{3}H]ryanodine binding results.

Recently there have been reports which demonstrate the coexistence of two isoforms of the ryanodine receptor in the same tissue type [12,36,12]. Previous studies carried out on bullfrog skeletal muscle identified two different isoforms (α and β) of the ryanodine receptor in the same cell [37]. Further characterisation indicated that the primary structure of the α isoform was highly homologous (80%) to the RyR1 from rabbit skeletal muscle while the β isoform is more than 85% identical to the RyR3 from rabbit brain. Takeshima and co-workers have developed a mouse model which contains a targeted mutation in the skeletal muscle ryanodine receptor gene [36]. The mutant muscle appears to lose its contractile response to electrical stimulation under physiological conditions but the Ca^{2+} release response to caffeine is retained, which implies that another RyR other than the skeletal type contributes to Ca^{2+} release in the mutant muscle. Ledbetter [12] selected primers to the 5′ end of the RyR1, RyR2 and RyR3 cDNA sequences and amplified each of the isoforms by RT-PCR protocols to demonstrate the expression of two RyR isoforms in each type of porcine striated muscle and all RyR isoforms in a number of regions of the nervous system. In the uterus it is possible that at various stages of pregnancy different amounts of each isoform may be differentially expressed or regulated. If one isoform were to be more efficient in mobilising intracellular Ca^{2+}, this manipulation of isoforms may alter the responsiveness and hence contractility of the intact tissue. The balance between possible RyR isoforms and other intracellular Ca^{2+} release receptors in myometrial smooth muscle, i.e. the $InsP_3$ receptor, may play an important role in maintaining the uterus in a quiescent state during pregnancy until early labour when the levels of important regulatory proteins are increased, e.g. gap junctions, ion channels, prostaglandin and oxytocin receptors.

This work may be the first indication of dual expression of ryanodine receptor isoforms in human smooth muscle tissue but the possibility cannot be excluded that the samples and cultures of human myometrium contain at least two different cell types each expressing a single isoform of the ryanodine receptor/channel complex. However, morphological analysis and antibody staining for α-actin indicate that the cultures are exclusively smooth muscle and not a mixed cell population.

The work presented here has demonstrated for the first time evidence for the co-expression of at least two RyR isoforms and the presence of a ryanodine-binding protein from human myometrial smooth muscle. The current methods do not allow any quantitative assessment of the amount of mRNA transcripts present or in what proportions these different isoforms may occur. Until this is known, the detailed physiological significance for the expression of two isoforms in the same cell remains obscure.

Acknowledgements: We gratefully acknowledge financial support from the British Heart Foundation, The Physiological Society, The Wellcome Trust, BBSRC Grant GR/H/52054 (to H.K.L.) and NIH Grant HL 27430 (to G.M.).

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